EFFECTS OF OMEGA-3 FATTY ACIDS ON RODENT MODELS OF BIPOLAR DISORDER AND ALCOHOLISM

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LIST OF ABBREVIATIONS

AA	arachidonic acid
CFG	Convergent Functional Genomics
DBP	D-box Binding Protein
EPA	eicosapentaenoic acid
KO	knockout
NST	non-stressed
PFC	prefrontal cortex
PUFA	polyunsaturated fatty acid
QTL	Quantitative Trait Loci
ST	stressed
TG	transgenic
WT	wild-type

CHAPTER I: INTRODUCTION AND BACKGROUND

Bipolar Disorder

Bipolar disorder is a debilitating illness that affects more than 3 million adult Americans [1]. People with this disease experience severe fluctuations in mood, from a very high-energy, manic state to a low-energy, depressed state. During manic states they are known to become impulsive and highly irritable, and experience racing thoughts, excessive talkativeness and decreased sleep. Sometimes suddenly they can transition to a depressed state with symptoms including increased feelings of hopelessness and sorrow, lack of energy, increased sleep, and thoughts of suicide. Indeed, lifetime incidence of suicide for bipolar patients is between 10-20% [2]. There are subsets of the disorder, with bipolar I consisting of repeated cycles of depressive and manic episodes, and bipolar II consisting of depressive and hypomanic episodes [2]. Episodes can last for days, months, or even years [1]. Bipolar disorder usually develops in late adolescence and early adulthood and without treatment can persist until death. This disorder also has a high rate of co-morbidity with alcoholism, leading to further destructive behavior [3].

The mechanisms underlying bipolar disorder are currently unknown. However, research has shown that this disorder is correlated with dysregulation in the dopamine and serotonin systems, as well as with pathology in the brain systems associated with regulating emotions [4]. When there is an excess of dopamine or serotonin, manic behavior, such as excessive talkativeness, impulsivity, irritability, and less need for sleep, is induced. When there is a deficiency in these amines, depressive mood and behavior, such as excessive need for sleep, and feelings of sorrow, helplessness, and worthlessness, are induced [1]. Other studies suggest that upregulation of the arachidonic acid (AA)

cascade is involved in the development of bipolar disorder. AA is an essential long-chain polyunsaturated fatty acid (PUFA), specifically an omega-6 fatty acid, that is involved in brain signaling via sertonergic, glutamatergic, dopaminergic, and cholinergic receptor stimulation [5]. Upregulation of the AA cascade can induce inflammation and neurological dysfunctions which have been implicated in bipolar disorder [6].

Drug treatment for bipolar disorder has been employed since the early 1950s with the discovery that lithium could stabilize mood [7]. Currently, drugs such as lithium, valproate, and carbamazepine are used to treat the symptoms of bipolar disorder. Interestingly, it has been shown that these drugs modulate the AA turnover rate in the brain. The AA cascade begins with phospholipase A_2 (PLA_2) hydrolyzing esterified AA from membrane phospholipids [5]. Long-chain acyl-CoA synthetases (Acsls) and acyl transferase reincorporate a majority of the released AA into brain phospholipids, while enzymes such as cyclooxygenases (COX) convert a small amount of the released AA into eicosanoids. Using radiolabeled fatty acid and quantitative autoradiographic analysis, incorporation rates of plasma unesterified AA into brain phospholipids can be determined in rodents [8]. Using these techniques, Chang et al. showed that chronic intake of lithium decreased AA turnover within the brain of rats by decreasing brain COX-2 activity and inhibiting the transcription of $cPLA_2$, which is the AA-selective calcium-dependent cytosolic form of PLA_2 [9]. Likewise, carbamazepine also decreases the turnover rate of AA in the rodent brain by decreasing COX-2 activity and inhibiting transcription of $cPLA_2$ [10]. Valproic acid also decreases the turnover rate of AA in the rodent brain, however, it appears to do so by inhibiting Acsl [11] and prevents nuclear factor-κB (NFκB) from inducing the transcription of COX-2 [12]. Figure 1, courtesy of Rao et al.,

summarizes the mode of action of lithium, carbamazepine, and valproic acid to affect turnover rates of AA [5]. Though there is strong evidence for the modulation of the AA cascade, other mechanisms of action for mood stabilizers have been postulated that include the involvement of inositol depletion, glycogen synthase kinase-3, protein kinase C, mitochondrial dysfunction and neurotrophins to name a few [13]. Mood stabilizing medication, though highly effective in reducing the symptoms of bipolar disorder, can cause many side effects including gastrointestinal pain, diarrhea, tremor, nocturnal urination, weight gain, oedema, flattening of affect, exacerbation of psoriasis, problems with memory, vigilance, reaction time and tracking, and have even led to death in some patients [14]. Prescription of these drugs for children and adolescents with bipolar disorder or who are at high-risk for developing the disorder is controversial, as the risk for experiencing adverse side effects is higher for these individuals [15]. In addition, it has been shown that mood stabilizing medication can cause birth defects in babies born to women on these medications during their pregnancy, and they can be transferred to the baby through breast milk [14, 16]. Thus, an alternative approach is needed to help stabilize the mood of bipolar patients, especially adolescents and pregnant women, that will not cause harm or negative side effects.

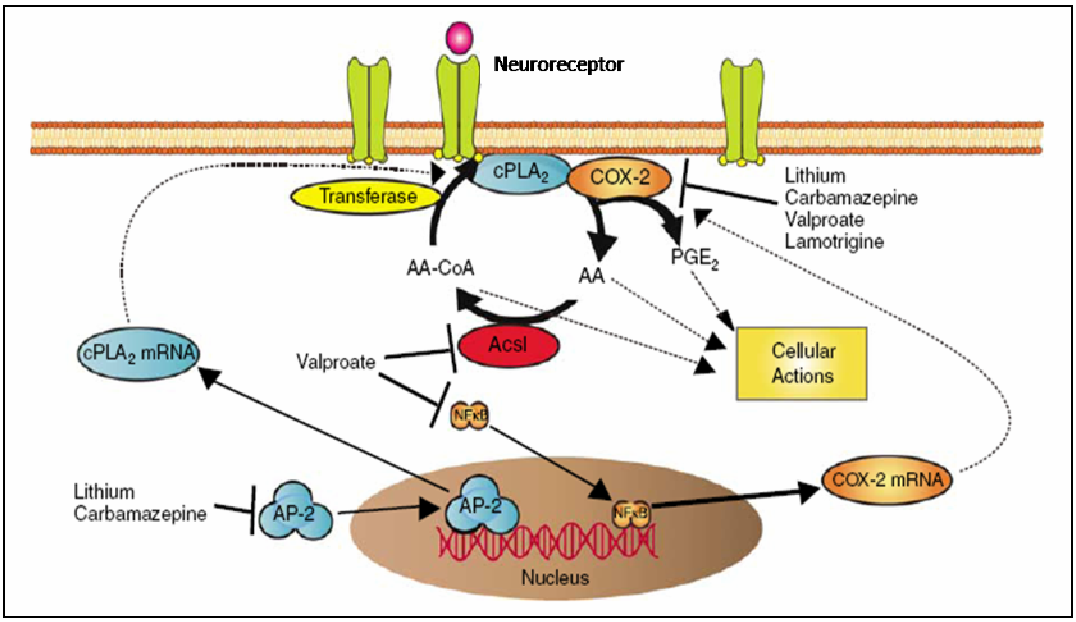

Figure 1. The arachidonic acid (AA) cascade and how drugs prescribed to fight bipolar disorder modulate the cascade. Specifically, lithium, carbamazepine, and valproate were shown to attenuate the cascade (Figure from Rao et al., 2008 [5]).

Omega-3 Fatty Acids

Omega-3 fatty acids, a subset of PUFAs, are essential nutrients that are involved in normal brain development and influence behavior and mood [17]. Currently, omega-3 fatty acid supplements are given to minimize the symptoms of rheumatoid arthritis [18] and coronary heart disease [19]. Preclinical trials of omega-3 fatty acid supplements have shown benefits for patients with autism, dyslexia, aggression and mood disorders [17]. Examples of key omega-3 fatty acids potentially involved in regulating mood include eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Indeed, studies by McNamara et al. found that erythrocyte DHA levels were 32% lower in bipolar patients compared to age-matched healthy controls [20], and patients with bipolar disorder had significant deficits (-24%) in DHA levels in postmortem prefrontal cortex (PFC) as compared to normal controls [21]. In addition, a recent study has shown that a diet deficient in omega-3 fatty acids is correlated with a heightened risk of developing mood disorders, such as bipolar disorder [22]. Omega-3 fatty acids are primarily found in fish oils, and interestingly, a study of 12 different countries showed that greater seafood consumption predicted lower lifetime prevalence rates of bipolar I disorder, bipolar II disorder, and bipolar spectrum disorder [23]. Several preliminary clinical trials have reported significant, beneficial results for patients with various psychotic disorders who had been given chronic omega-3 fatty acid supplements [24-26].

The exact mechanism by which omega-3 fatty acids operate in bipolar patients is presently unknown. However, fluctuations in the concentrations and ratios of long-chain PUFAs, specifically AA and DHA have been linked to bipolar disorder [5]. As mentioned in the previous section, mood stabilizers have been shown to downregulate the

AA metabolic cascade in rodents. In these same rodents, the mood stabilizers did not affect the DHA cascade [5]. However, when a diet low in omega-3 fatty acids is given to rats for 15 weeks, the brain AA metabolic cascade is upregulated, while the DHA cascade is downregulated [27]. Increased levels of prostaglandins derived from AA cause inflammation and have been linked to neurological diseases. Interestingly, Stahl et al. proposed that omega-3 fatty acids reduce the level of these prostaglandins in humans [28]. Su argues that the benefit of eating fish with a high amount of EPA and DHA is that these fatty acids displace AA from the cell membrane phospholipid pool [6]. As the amount of AA turnover is increased in bipolar patients compared to healthy individuals [2], this shift may be beneficial for these patients. Piomelli et al. also suggest that DHA works by interacting with dopaminergic or sertonergic receptors altered in bipolar patient brains to modulate receptor-coupled AA release [29]. Figure 2 summarizes the relationship between DHA, EPA and AA and the outcomes of fluctuations of these fatty acids (Figure courtesy of Su 2009 [6]). Overall, studies show a connection between decreased levels of omega-3 fatty acids in humans and increased rates of bipolar disorder, whereas increased levels of these fatty acids in the brain and blood may be beneficial for these patients.

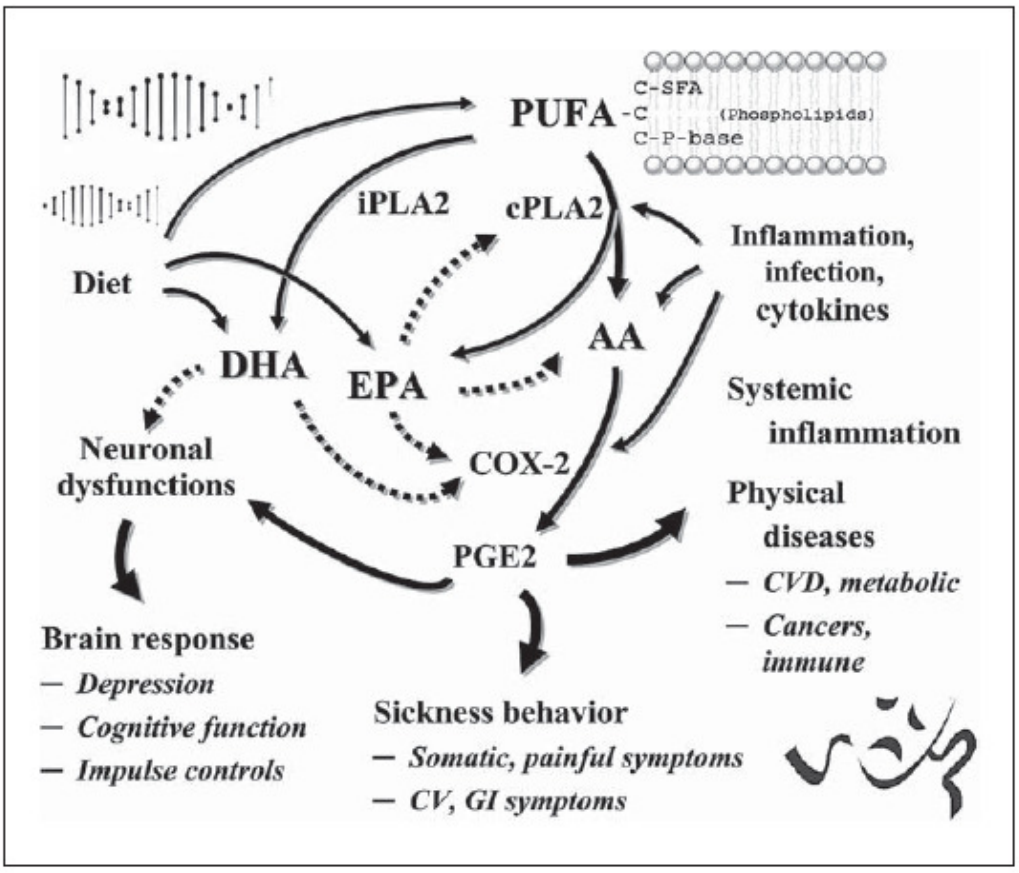

Figure 2. Genetic and environmental factors related to omega-3 fatty acid and omega-6 fatty acid involvement in biological processes and dysfunctions. The omega-3 fatty acids (DHA and EPA) cause the downregulation of factors that induce neuronal dysfunction, whereas the omega-6 fatty acid, arachidonic acid (AA), appears to induce inflammation and neuronal dysfunction (Figure from Su 2009 [6]).

Previous Studies

Our lab had previously identified the D-box Binding Protein (DBP) as a potential candidate gene for inducing bipolar disorder [30-31] using Convergent Functional Genomics (CFG), which cross-matches various lines of evidence from published scientific literature to determine potential candidate genes for psychiatric disorders [30, 32-34]. This method is described in more detail in the following section. DBP is a circadian clock gene that has been mapped on chromosome 19q13 near a human genetic linkage locus for depression [35] and bipolar disorder [36]. Circadian clock genes have been associated with bipolar disorder, specifically the switch from depression to manic states [30, 37-38]. This association is particularly observed as sleep abnormalities, as bipolar patients will often feel a need for excessive sleep during their depressed phase, but then experience a reduction in need for sleep during the manic stage [39]. Indeed, sleep deprivation is often suggested as an initial treatment of severe depression, whereas, it can lead to an accelerated switch to a manic episode in patients with bipolar disorder [40-41]. Franken et al. found that DBP knockout (KO) mice have abnormal circadian rhythms as well as abnormal sleep regulation [42]. To further study the potential role of DBP in bipolar disorder and related disorders, our laboratory conducted analyses of behavior and gene expression in mice with a constitutive homozygous deletion of DBP (DBP KO mice) [31]. Blood and brain gene expression studies were conducted to identify genes that change concurrently, and subsequently may provide candidate biomarkers for the disease [43].

Similar to sleep deprivation, stress can be the catalyst to induce a switch to a manic episode in bipolar patients [40]. For this reason, in our previous study DBP KO

and WT mice were stressed for a period of 4 weeks and the outcomes of the open-field locomotion test were compared to non-stressed DBP KO and WT mice. To induce chronic stress, mice were single-housed and underwent a stress paradigm consisting of forced swim, tail flick, and tail suspension test to induce acute stress. The day prior to the stress paradigm, the mice were also sleep deprived by keeping lights off (as mice are nocturnal) for a complete 24 hours, as sleep deprivation in humans can cause depression symptoms in healthy adults and enhance the switch to manic states in bipolar patients [40]. A week after the stress paradigm, the open-field test was administered to track movement.

The open-field test is used to measure exploratory behavior and locomotor activity in rodents. Using this technique, one can discover motor deficits and anxiety-related behavior. The open field is typically a square enclosure with high walls to prevent escape. This box is divided into nine regions: four corners, four side squares, and one center square. A video camera is placed above the box to capture images that are analyzed with computer software to determine the amount of time the rodent spends in motion or resting, the velocity of movement, and the region in which the rodent is moving after it is placed in the square enclosure. Visual tracking files of the rodent movement are also saved for each trial, with a trial lasting for 30 min in our studies. This test can be used to measure anxiety or impulsivity by comparing the amount of time spent in the center for a test rodent compared to control. Rodents instinctively spend little time in the center region as they are more prone to attack out in the open. Mice or rats that spend significantly more time in the center than control may be more impulsive, whereas

rodents that spend significantly less time in the center and move less in general than control may be more anxious [31].

In our previous study, non-stressed mice were group housed, did not undergo the stress paradigm, and were not sleep deprived. Total distance traveled was determined and non-stressed wild-type mice (NST WT), non-stressed DBP KO mice (NST KO), stressed wild-type mice (ST WT), and stressed DBP KO mice (ST KO) were compared. At baseline, NST KO mice exhibited an overall decrease in the total distance traveled as compared to NST WT mice. This effect was reversed with treatment of the stimulant, methamphetamine, which has been used in rodent studies to model mania [44]. The observed significant decrease in baseline locomotion of NST KO mice compared to NST WT mice, along with the reported sleep EEG abnormalities in these KO mice [42], suggests that the NST DBP KO mice phenotypically resemble the depressive phase of bipolar disorder.

When DBP KO mice were subjected to chronic and acute stress they showed a diametric switch in their locomotion compared to ST WT mice. ST KO mice were hyperlocomotive, while ST WT mice were hypolocomotive (Figure 3a and b), and this difference was minimized with administration of methamphetamine (Figure 3a). This switch in locomotion resembles the switch from a depressed episode to a manic stage when bipolar patients endure stress and/or sleep deprivation [40, 45].

If the change in locomotion seen in NST KO mice compared to sleep deprived ST KO mice was related to an endophenotype that is related to bipolar disorder, then pre-treatment of a mood stabilizer should adjust this behavioral response. Indeed, sleep deprivation induced a significant increase in locomotion for DBP KO mice, and pre-

treatment with valproate prior to testing brought this elevated locomotion level back to non-sleep deprived levels (Figure 3c).

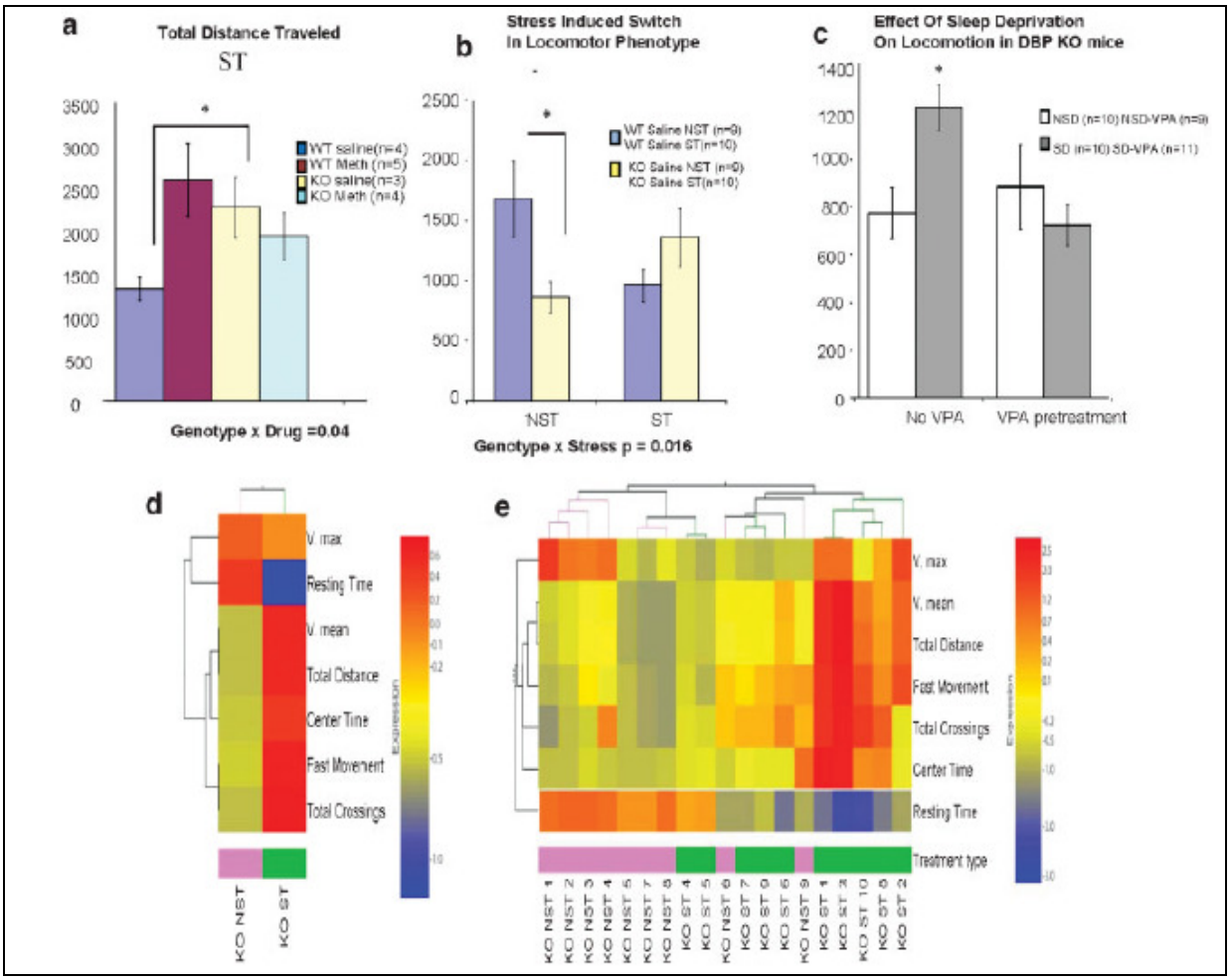

Figure 3. Phenomics of DBP KO ST mice: locomotion, switch, sleep deprivation, clustering. (a) Results of open-field test in both wild-type and knockout mice with and without methamphetamine treatment (*p-value = 0.037); (b) stress-induced switch in total distance traveled (*p-value = 0.016); (c) sleep deprivation caused an increase in the total distance traveled by DBP KO mice that was modulated with valproic acid pretreatment. SD – sleep deprived, NSD – non-sleep deprived, VPA – valproic acid (*p-value = 0.007); (d) group, and (e) individual mice clustering of ST – stressed mice and NST – non-stressed mice. V. max – maximal velocity, V. mean – mean velocity, red – increased, blue – decreased (Figure from Le-Niculescu et al., 2008 [31]).

To further show the extremes of behavior between the ST KO and NST KO mice, a two-way hierarchical clustering of the mouse locomotor behavioral data measures (phenes; Figure 3d,e) [46] was created using GeneSpring. Z scores were determined using results from each mouse for the different phenes and were put into the program instead of the usual use of gene expression intensity numbers, allowing for the visual clustering of phenes. The figure shows that the mice in each group clustered for the most part into two distinct groups. A point of interest from this clustering analysis is the contrast between ST and NST KOs in the amount of time spent in the center square, "Center Time." ST DBP KO mice spent more time in the center of the field than their NST counterparts. This may indicate an increase in risk-taking, impulsive behavior, which is often observed in bipolar patients in the manic phase [47]. The phene with the strongest contrast between groups is Resting Time. ST KO mice took much less time to rest than NST KO mice, which is analogous to increased activity during the manic stage of bipolar disorder compared to the decreased activity during the depressed stage.

As previously mentioned, there is a high degree of co-morbidity of alcoholism with bipolar disorder [3, 48]. For this reason, along with the fact that DBP was identified as a potential candidate gene for alcoholism using the CFG approach [34], our lab studied the amount of alcohol consumption by male and female DBP KO mice. It was hypothesized that alcohol intake would be increased in DBP KO mice, specifically ST KO mice, as they may have an increased hedonic impulse to drink more alcohol than ST WT counterparts. Interestingly, at baseline during the beginning of the chronic stress paradigm (single-housing), ST KO mice drank less alcohol than ST WT mice, however, they exhibited a switch in response to the stress and consumed more alcohol over a 30-

day period compared to ST WT mice (Figure 4). With these promising results, along with interesting genetic expression studies described in Le-Niculescu et al., 2008 [31], we decided to perform further tests on DBP KO mice, as a potential model of bipolar disorder.

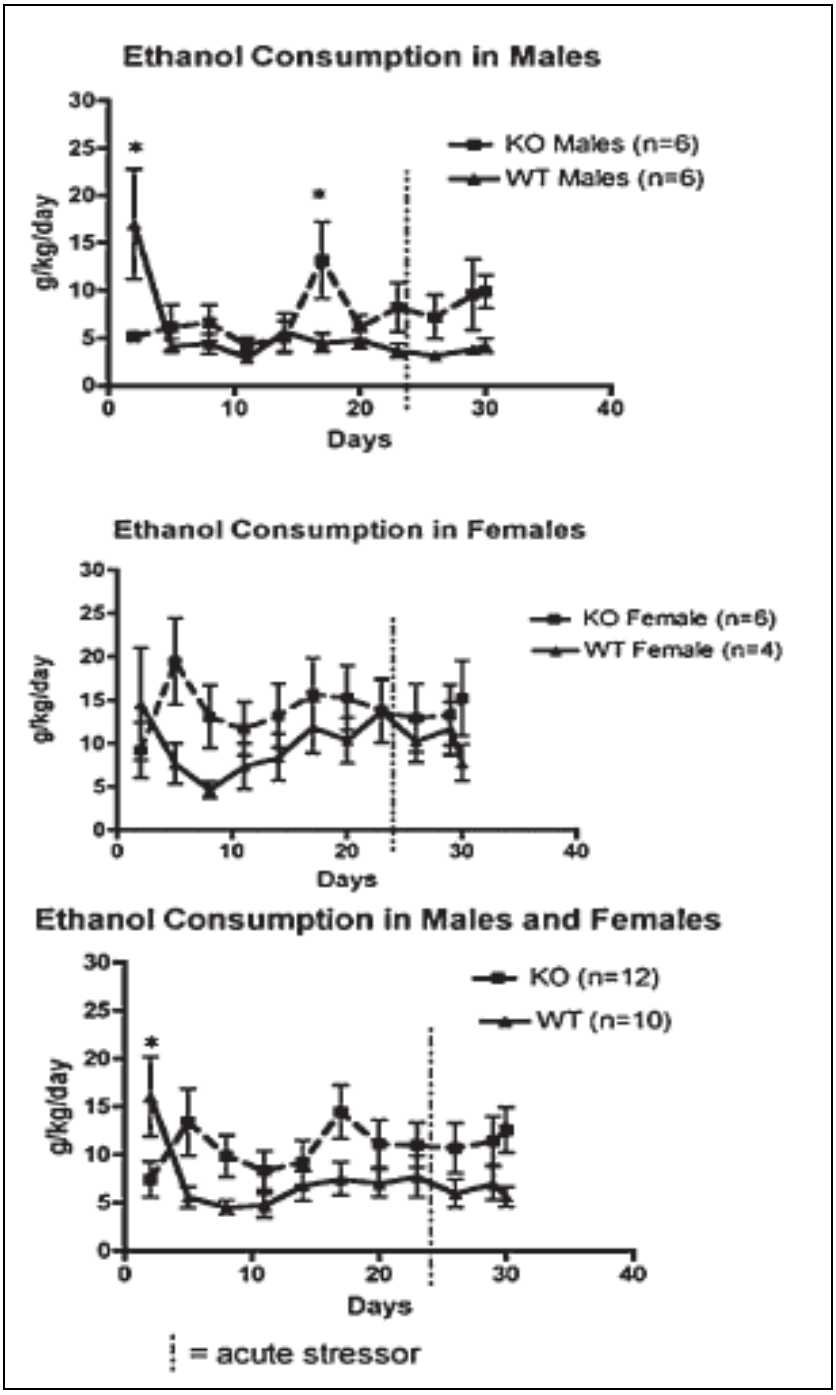

Figure 4. Ethanol consumption during the stress paradigm in DBP KO and WT mice. Dotted vertical line indicates acute stress paradigm was performed on that day; * indicates p-value < 0.05 (Figure from Le-Niculescu et al., 2008 [31]).

Background of Present Study

In the following manuscript we used several behavioral and bioinformatic techniques to evaluate the differences between the mice in our study. Specifically, the forced swim test, open-field video tracking test, microarrays, and the CFG approach were used to evaluate behavior in the mice and analyze the genetic information obtained from their brains and blood. A brief synopsis of the reasons these specific tests were employed, including their potential benefits and limitations, follows.

Presently, bipolar disorder patients are diagnosed by describing their mood, habits, and feelings to a psychiatrist. Since one cannot interview a rodent to determine their mood, behavioral tests, which reveal differences in behavior as a result of the changes in mood, are necessary. The forced swim test has been implemented since the 1970s as a way to determine learned helplessness or behavioral despair in rodents and to test the effects of antidepressants on reversing this behavior [49]. In their studies on memory, Porsolt et al. noticed that in the water maze memory test some of their rodents would give up and simply float instead of looking for the exit, leading the researchers to propose that these rodents might have been displaying signs of despair. To test this, Porsolt dropped mice into a cylinder filled with water for six minutes and immobility was recorded for the last four minutes. The group tested the effects of antidepressants on mice using this method and found that mice on antidepressants were more mobile than the mice without the drugs, allowing them to conclude that this test could provide a measure of depression in rodents. In the intervening time, this model, with some modifications, has been employed in numerous studies to evaluate the effectiveness of potential antidepressants and decipher behavioral differences between wild-type and knockout

mice [50]. A potential flaw of this test is that the immobility seen in mice may not be representative of depressed mood, but could instead be an attempt of the mouse to conserve energy in an inescapable situation. Thus, immobility could be considered an adaptive behavior, which could represent successful coping skills, instead of behavioral despair [50]. Also, the outcome of this test is reliant on the scoring ability of the observer. This can lead to discrepancies between scores for different laboratories. For these reasons, this test should not be the only measure of behavioral depression employed in rodents. Instead, forced swim can provide preliminary evidence of differences between different genetic or treatment groups, and researchers should include other behavioral and biochemical tests to validate the observed outcomes. For this reason, we also employed the open-field test to measure locomotion and impulsivity in our mice, and gene expression studies to examine the genetic differences between our mice.

The standard protocol for the open-field test was described in detail in the previous section. For this test, the most used outcome measure is locomotion, however, this outcome can be affected by motor output, exploratory drive, fear-related behavior, illness, circadian cycle, and many other factors [51]. Because of this, researchers must supplement this test with other analyses to make conclusions about the outcomes. For this reason, we employed genetic studies to determine differences among different treatment and genetic groups.

To determine the up or downregulation of the genes between groups, we removed and sectioned the brain, took blood samples, and tested mRNA expression in the blood and PFC using the Affymetrix GeneChip Mouse Genome 430 2.0 Array and analyzed hybridization using Affymetrix Microarray software. Microarrays were first developed in

the late 1990s and are used to detect gene expression levels in cells or tissue by measuring the hybridization of mRNA from the samples to tens of thousands of known genes that have been attached to a glass surface [52]. Outputs of changes from microarrays can be tens of thousands of genes; for this reason, we used a p-value threshold of 0.0025 to reduce the output list and to select the most significantly changed genes from the list.

Microarrays have their limitations. For example, mRNA can be degraded and lead to false data if tissue has not been processed rapidly. Likewise, tissue samples are a mixture of different cell types thus leading to the possibility of a depression of signal-to-noise as upregulated genetic material in one cell type may be downregulated in other cell types [53]. For these reasons, we flash froze brain tissue and preserved the blood samples in PAXgene blood RNA collection tubes to prevent mRNA degradation, and we sliced the brains into regions, using only the PFC region for microarray analysis. A limitation of our approach is that microarrays were our only method for detecting gene expression changes thus were possibly accompanied by biological or technical errors, as our experiments and microarray analysis require many steps [31, 53]. Knowing this, we designed our study to minimize false positives by comparing gene expression data using compiled total RNA extracted from the brain tissue samples or blood from three mice per treatment and genetic group from experiments performed at three different times. We only evaluated the genes that were reproducibly changed in the same direction in at least six out of nine independent comparisons. This way, we could be more confident of the genes that were shown to be significantly different between groups. Although we used methods to reduce the number of genes on the original output list, the number of genes on

the remaining list can be quite extensive. For this reason we employed the CFG approach to prioritize the list of genes.

CFG is an approach that prioritizes the hundreds to thousands of genes that are shown to be significantly changed between groups in microarrays [54]. Using multiple lines of published evidence for a disease and related diseases, the genes are ranked according to their score based on the number of different lines of evidence in which they have been associated to the disease. The lines of evidence for this study include human genetic linkage or association evidence, human postmortem brain evidence, human blood evidence, and mouse genetic evidence, either Quantitative Trait Loci (QTL) or transgenic (TG). Once the genes are ranked based on the CFG score, we organize the genes into a pyramid as a visual representation of the scoring of the genes and the lines of evidence with which they are affiliated, with the highest ranking genes at the top. By cross-matching multiple lines of independent evidence, more confidence can be placed on the gene and its role in the disease, leading to determination of higher probability candidate genes, blood biomarkers and potential mechanisms and pathways for the disease. This approach has its limitations, as it is reliant on published data, and key genes that have yet to be linked to the disease may receive an artificially low score or go unnoticed altogether. In this regard, CFG will progress in its accuracy as significant and thorough published scientific research progresses. Our hope is that CFG will only get better at determining candidate genes and biomarkers with time and the progression of the field.

CHAPTER II: MANUSCRIPT

Omega-3 fatty acids as a treatment for psychiatric disorders: phenomic, genomic and blood biomarker studies in animal models

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Running title: Omega-3 fatty acids, bipolar disorder and alcoholism

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Introduction

We have previously identified the clock gene D-box Binding Protein (DBP) as a potential candidate gene for bipolar disorder [54], alcoholism [34], and schizophrenia [55] using a Convergent Functional Genomics (CFG) approach. In follow-up work [31], we reported that mice with a homozygous deletion of DBP have lower locomotor activity, blunted responses to stimulants, and gain less weight over time. In response to a chronic stress paradigm, the mice exhibit a diametric switch in these phenotypes. DBP knockout (DBP KO) mice are also activated by sleep deprivation, similar to bipolar patients, and that activation is prevented by treatment with the mood stabilizer drug valproate. Moreover, these mice show increased alcohol intake following exposure to stress. Microarray studies of brain and blood revealed patterns of gene expression changes that may explain the observed phenotypes. CFG analysis of the gene expression changes identified a series of novel candidate genes and blood biomarkers for bipolar disorder, alcoholism and stress reactivity.

Omega-3 fatty acids (eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) are essential fatty acids, DHA being the final metabolic pathway compound. They have been described to have mood and psychosis modulating properties, in both preclinical models and some clinical trials. For example, deficits in omega-3 fatty acids have been linked to increased depression and aggression in animal models [56] and humans [57]. Of note, deficits in DHA have been reported in the postmortem orbitofrontal cortex of patients with bipolar disorder, and were greater in those that had high verses low alcohol abuse [21]. Omega-3 fatty acids have been reported to be clinically useful in the treatment of both mood [58-61] and psychotic disorders [62-64].

To date, there is no clear understanding of how omega-3 fatty acids work in terms of psychotropic effects, or indeed how effective they are. Unlike most psychiatric drugs, these natural compounds have minimal side-effects, and intriguing evidence for favorable health benefits [65]. Particularly for female patients of child-bearing age, the potential fetus-harming side-effects of mood stabilizing and antidepressant medications are a major issue. As such, if the action of omega-3 fatty acids in mood disorders and related disorders could be substantiated by understanding their mechanistic effects, they might become an important addition to the therapeutic armamentarium of psychiatrists and primary care doctors.

Based on the above, we decided to test omega-3 fatty acids, specifically DHA, as a way of reversing the phenotypic, gene expression and biomarker abnormalities present in this new model (DBP KO mice). We also studied the effects of DHA on modulating alcohol consumption in DBP mice and in an independent animal model, the alcohol-preferring (P) rat, a well established model of alcoholism. The work described has important translational implications for understanding and validating a new treatment approach, that follows the Hippocratic principle of “first do no harm” and may favorably impact multiple co-morbid conditions.

Materials and Methods

Mouse Colony

The generation of transgenic mice carrying the DBP KO has been previously described in detail [31]. DBP (+/-) heterozygous (HET) mice were bred to obtain mixed littermate cohorts of DBP (+/+) wild-type (WT), HET and DBP (-/-) knockout (KO) mice. Mouse pups were weaned at 21 days and housed by gender in groups of two to four in a temperature- and light-controlled colony on reverse cycle, with food and water available *ad libitum*. DNA for genotyping was extracted by tail digestion with a Qiagen DNeasy Tissue kit, following the manufacturer's protocol for animal tissue (Qiagen, Valencia, CA). The following three primers were used for genotyping by polymerase chain reaction (PCR):

Dbp forward: TTCTTTGGGCTTGCTGTTTCCCTGCAGA

Dbp reverse: GCAAAGCTCCTTTCTTTGCGAGAAGTGC (WT allele)

lacZ reverse: GTGCTGCAAGGCGATTAAGTTGGGTAAC (KO allele)

WT or KO mice 8 to 12 weeks old were used for experiments.

Animal Housing, Diets and Treatment

All mice were housed for at least one week prior to each experiment in a room set to an alternating light cycle with 12 hours of darkness from 10 a.m. to 10 p.m., and 12 hours of light from 10 p.m. to 10 a.m. At the start of the experiment, male and female DBP WT, or DBP KO mice were placed one of two diets: (1) low DHA custom research diet (TD. 00522 Harlan Teklad, Madison, WI), a DHA-depleted low omega-3 PUFA test diet adequate in all other nutrients (n-6/n-3 ratio of 85:1 with 6% fat as safflower oil) [66]; or (2) high DHA custom research diet (TD. 07708 same as the low DHA diet, except

supplemented with 0.69% Algal DHA; Martek Bioscience, Columbia, MD) [66]. The DBP mice were fed the low DHA diet (0% DHA), or high DHA diet (0.69% DHA) for 28 days. Mice, food, and water were weighed twice a week. Water was refilled once a week.

During the 28 day period, the mice were single-housed to induce chronic stress, and underwent behavioral challenge tests on day 21 of the experiment to induce acute stress. The behavioral challenge tests consisted of sequential administration of the forced swim test, tail flick test, and tail suspension test. At four weeks (day 28) the mice were injected with saline to keep handling consistent with previous work [31] and open-field locomotor activity was assessed with SMART II video-tracking software. After video-tracking, brain and blood were harvested as previously described [31] for use in microarray studies.

Behavioral Challenge Tests

Forced Swim Test

Forced swim test (FST) experiments were performed on day 21 of treatment during the dark cycle. Mice were placed in a transparent Plexiglas cylinder (64 cm height x 38 cm diameter), with water depth of 30 cm and temperature of 23±2°C. Water was replaced after each mouse. Time spent immobile in a 10 minute interval was scored live by two independent observers blinded to the animal's genotype and treatment group.

Tail Flick

Immediately following the FST, the mice were dried with paper towels and placed in the Plexiglas chamber of the Tail Flick Analgesia Meter System (San Diego Instruments, San Diego, CA). The mouse's tail was placed over a window located on the

Tail Flick platform where a light beam shines to heat the tail at a reliable, reproducible rate for 20±1 sec. This test was performed as an acute stressor, and not as a way to measure the mouse's response to pain, as it is confounded by the preceding FST.

Tail suspension

For the third part of the acute stress paradigm, the mouse was suspended by its tail, approximately 30 cm above the ground for 5 minutes. This test was also performed as an acute stressor, and not as a way to measure the mouse's behavior, as it is confounded by the preceding tests.

Locomotion Pattern Testing

A SMART II Video Tracker system (San Diego Instruments, San Diego, CA) under normal light was used to track movement of mice. The mice were placed in the lower-right-hand corner of one of four adjacent, 41x41x34 cm^3 enclosures. Mice were not allowed any physical contact with other mice during testing. Each enclosure had nine pre-defined areas, that is, center area, corner area, and wall area. The movements of the mice were recorded for 30 minutes. The enclosures were cleaned with ethanol and water after each tracking. Measures of total distance traveled, center entry, center time, fast movement, resting time, average velocity (V mean) and maximum velocity (V max) were obtained.

Alcohol Consumption Experiments in DBP KO Mice

To create an alcohol free-choice drinking paradigm, male WT and DBP KO mice were placed in individual cages with both a bottle of ~250 ml cold tap water and a bottle of ~250 ml 10% v/v ethanol, and either the low or high DHA diet for 28 days, with an acute stressor (behavioral challenge tests described above) on day 21. The amount of

ethanol and water consumed was recorded twice a week, at which time the location of the bottles was switched to prevent positional bias. The bottles were refilled with fresh solution once a week. Following 28 days of free-choice drinking, the animals were injected with saline and their locomotor activity was assessed with SMART II video-tracking software. After video-tracking, the microsurgery was performed to separate brain into regions and blood of each animal was harvested for use in future microarray studies.

Alcohol Consumption Experiments in Alcohol-Preferring (P) rats

Experimentally naïve, male alcohol-preferring (P) rats, 4-6 months of age at the start of the experiment, were used as subjects. They were placed on three diets: (1) low DHA custom research diet (described above); (2) high DHA custom research diet (described above); and (3) normal rat chow (7001 Harlan Teklad, Madison, WI) for a duration of 28 days. Food and water were available *ad libitum* throughout the experiments. Rats were given continuous free-choice access in the home cage to 15% v/v ethanol and water. Alcohol intake was measured daily throughout the experiment.

Behavioral Statistical Analysis

Behavioral data are expressed as the mean and ± S.E.M. Two-way analysis of variance (ANOVA) was used to determine statistically significant differences for factors of gender, genotype, and diet, using SPSS statistical software (SPSS Inc., Chicago, IL). It was determined a priori based on previous research to use one-tailed, two sample independent t-tests assuming unequal variance to determine significant differences between individual groups. Differences between groups were considered significant at $p \leq 0.05$.

RNA Extraction and Microarray Work

Following the open-field locomotor test, mice were sacrificed by cervical dislocation, decapitated and brains and blood were harvested. Behavioral testing and tissue harvesting were done at the same time of day in all experiments. Once the brains of the mice were harvested, they were stereotactically sliced, and hand micro-dissected using Paxinos mouse anatomical atlas coordinates, to isolate anatomical regions of interest [32, 55]. Tissues were flash frozen in liquid nitrogen and stored at -80°C pending RNA extraction. Approximately 0.5-1.0 ml of blood per mouse was collected into a PAXgene blood RNA collection tubes (BD diagnostic; www.VWR.com). The PAXgene blood vials were stored at -4°C overnight, and then at -80°C until future processing for RNA extraction.

Standard techniques were used to obtain total RNA (22 gauge syringe homogenization in RLT buffer) and to purify the RNA (RNeasy mini kit, Qiagen, Valencia, CA) from micro-dissected mouse brain regions. For the whole mouse blood RNA extraction the PAXgene blood RNA extraction kit (PreAnalytiX, a Qiagen/BD company) was used, followed by GLOBINclear™–Mouse/Rat (Ambion/Applied Biosystems Inc., Austin, TX) to remove the globin mRNA. All the methods and procedures were carried out as per the manufacturer's instructions. The quality of the total RNA was confirmed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). The quantity and quality of total RNA was also independently assessed by 260 nm UV absorption and by 260/280 ratios, respectively (Nanodrop spectrophotometer). Starting material of total RNA labeling reactions was kept consistent within each independent microarray experiment.

Equal amounts of total RNA extracted from the PFC brain tissue samples or blood from 3 mice per group was pooled for each experimental condition and used for labeling and hybridization to Mouse Genome 430 2.0 arrays (Affymetrix, Santa Clara, CA). The GeneChip Mouse Genome 430 2.0 Array contains over 45,000 probe sets that analyze the expression level of over 39,000 transcripts and variants from over 34,000 well-characterized mouse genes. Standard Affymetrix protocols were used to reverse transcribe the messenger RNA and generate biotinlylate cRNA (http://www.affymetrix.com). The amount of cRNA used to prepare the hybridization cocktail was kept constant within each experiment. Samples were hybridized at 45ºC for 17 hours under constant rotation. Arrays were washed and stained using the Affymetrix Fluidics Station 400 and scanned using the Affymetrix Model 3000 Scanner controlled by GCOS software. All sample labeling, hybridization, staining and scanning procedures were carried out as per the manufacturer's recommendations.

Quality Control

All arrays were scaled to a target intensity of 1000 using Affymetrix MASv 5.0 array analysis software. Quality control measures including 3'/5' ratios for GAPDH and beta-actin, scaling factors, background, and Q values were used.

Microarray Data Analysis

Data analysis was performed using Affymetrix Microarray Suite 5.0 software (MAS v5.0). Default settings were used to define transcripts as present (P), marginal (M), or absent (A). A comparison analysis was performed for DBP KO mice on the high DHA diet, using DBP KO mice on low DHA diet mice as the baseline. "Signal," "Detection," "Signal Log Ratio," "Change," and "Change p-value," were obtained from this analysis.

An empirical p-value threshold for change of $p<0.0025$ was used. Only transcripts that were reproducibly changed in the same direction in two out of three comparisons were analyzed further.

Gene Identification

The identities of transcripts were established using NetAFFX (Affymetrix, Santa Clara, CA), and confirmed by cross-checking the target mRNA sequences that had been used for probe design in the Affymetrix Mouse Genome 430 2.0 arrays GeneChip® with the GenBank database. Where possible, identities of ESTs are established by BLAST searches of the nucleotide database. A National Center for Biotechnology Information (NCBI) (Bethesda, MD) BLAST analysis of the accession number of each probe-set was performed to identify each gene name. BLAST analysis identifies the closest known mouse gene existing in the database (the highest known mouse gene at the top of the BLAST list of homologues) which then could be used to search the GeneCards database (Weizmann Institute, Rehovot, Israel) to identify the human homologue. Probe-sets that did not have a known gene are labeled "EST" and their accession numbers kept as identifiers.

Human Tissue (Postmortem Brain, Blood) Convergence

Information about our candidate genes was obtained using GeneCards, the Online Mendelian Inheritance of Man database (http://ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM), as well as database searches using PubMed (http://ncbi.nlm.nih.gov/PubMed) and various combinations of keywords (gene name, bipolar, depression, alcoholism, stress, anxiety).

Human Genetic (Linkage, Association) Convergence

To designate convergence for a particular gene, the gene had to map within 10 cM (see [54] for detailed discussion) of a microsatellite marker for which at least one published study showed evidence of genetic linkage or a positive association study for the gene itself (for bipolar disorder, depression, alcoholism, stress, anxiety). The University of Southampton's sequence-based integrated map of the human genome (The Genetic Epidemiological Group, Human Genetics Division, University of Southampton: http://cedar.genetics.soton.ac.uk/public_html/) was used to obtain cM locations for both genes and markers. The sex-averaged cM value was calculated and used to determine convergence to a particular marker. For markers that were not present in the Southampton database, the Marshfield database (Center for Medical Genetics, Marshfield, WI, USA: http://research.marshfieldclinic.org/genetics) was used with the NCBI Map Viewer website to evaluate linkage convergence. We have established in the laboratory manually created databases of all the published human postmortem and human genetic literature to date on bipolar and related disorders. These large databases have been used in our CFG cross-validation analyses.

Mouse Genetic (QTL, transgenic) Convergence

To search for mouse genetic evidence – QTL (Quantitative Trait Loci) or transgenic (TG) – for our candidate genes, we utilized MGI_3.54 - Mouse Genome Informatics (Jackson Laboratory) and used the search menu for mouse phenotypes and mouse models of human disease/abnormal behaviors, using the following sub-categories: abnormal emotion/affect behavior, abnormal sleep pattern/circadian rhythm, addiction and drug abuse. To designate convergence for a particular gene, the gene had to map

within 10 cM of a QTL marker for the abnormal behavior, or a transgenic mouse of the gene itself displayed that behavior.

CFG Analysis Scoring

Genes that changed in the same direction in two out of three experiments in the brain or blood of DBP KO mice on the high DHA diet compared to mice on the low DHA diet were scored. Genes changed in the PFC received 1 point; genes changed in the blood received an additional 1 point. If the brain and blood direction of change was concordant, the gene received an additional 2 points. The other four cross-validating lines of evidence (human and animal model data) received a maximum of 1 point each (for human genetic data: 0.5 points if it was linkage, 1 point if it was association; for mouse genetic data: 0.5 points if it was QTL, 1 point if it was transgenic). Thus, the maximum possible CFG score for each gene was 8 points.

As we are interested in discovering brain-blood candidate biomarker genes of response to DHA, we weighted data from the DHA treated DBP KO ST mice more heavily, bringing the data from this one methodological approach on par with the data from all the other methodological approaches combined. It has not escaped our attention that other ways of weighing the scores of line of evidence may give slightly different results in terms of prioritization, if not in terms of the list of genes per se. Nevertheless, we feel this simple scoring system provides a good separation of genes based on our focus on identifying candidate genes for mood.

Pathway Analysis

Ingenuity 8.0 (Ingenuity Systems, Redwood City, CA) was employed to analyze the molecular networks, biological functions and canonical pathways of the top candidate genes resulting from our CFG analysis. The list of top genes was entered into the software and charts were made based on the provided results.

Results

Phenomic Studies: Behavioral Effects of DHA in DBP KO mice

In order to see the effects of dietary omega-3 fatty acid supplementation on our animal model, we tested the effects of a diet high in the omega-3 fatty acid, DHA, verses a diet low in DHA. We used a diet enriched in DHA, as the evidence for efficacy of EPA, the other main omega-3 fatty acid, is less encouraging [67]. Overall results for the forced swim test did not reach significance using two-way ANOVA (Figure 5a), however significance was reach for females when separating the mice based on gender (Figure 5c. Two-factor ANOVA p-value = 0.02). There was a significant difference in immobility time between ST WT and ST KO mice on low DHA diet (t-test p-value = 0.002), with ST WT mice swimming significantly less than ST KO mice. However, this significant gap between immobility times was reduced for female mice on the high DHA diet, as there was no longer a significant difference between ST WT and ST KO on this diet (t-test p-value=0.32). There was also a significant difference between ST WT mice on the high DHA diet compared to WT littermates on the low DHA diet (t-test, p-value = 0.02). There was a trend toward decreased swimming for ST KO mice on the high DHA diet compared to ST KO mice on the low DHA diet (t-test p-value = 0.09). Weight gain was not significantly different among groups (ANOVA p-value = 0.50, data not shown).

Although males did not show a significant difference among groups in the forced swim test, there were trends similar to that of female forced swim data (Figure 5e. ANOVA p-value = 0.62). Open-field video tracking revealed similar results regarding mobility to that of female forced swim data (Figure 5f. ANOVA p-value = 0.05). There was a significant difference in total distance traveled between ST WT and ST KO on the

low DHA diet (t-test p-value = 0.0002), with ST WT mice moving significantly less than ST KO mice. However, there was no significant difference in immobility time between ST WT and ST KO male mice on the diet high in DHA (t-test p-value = 0.08). Significance was also reached for the difference between ST KO mice on the high DHA diet compared to KO littermates on the low DHA diet (t-test p-value = 0.03). Weight gain was not significantly different among groups (ANOVA p-value = 0.77, data not shown). Female mice showed similar trends to males during video tracking, but did not reach statistical significance (Figure 5d. ANOVA p-value = 0.16).

Similar to our previous results for male ST DBP KO verses NST DBP KO male mice [31], the measure of resting time in the open-field test was most different between male ST KO mice on high (increased) verses low (decreased) DHA diet (data not shown). Likewise, center time (time spent in the center region of the open field), was decreased in mice on the high diet verses low DHA diet. A decrease in center time may correlate with a decrease in risk-taking behavior, as mice generally avoid the potentially dangerous, center area of an open field.

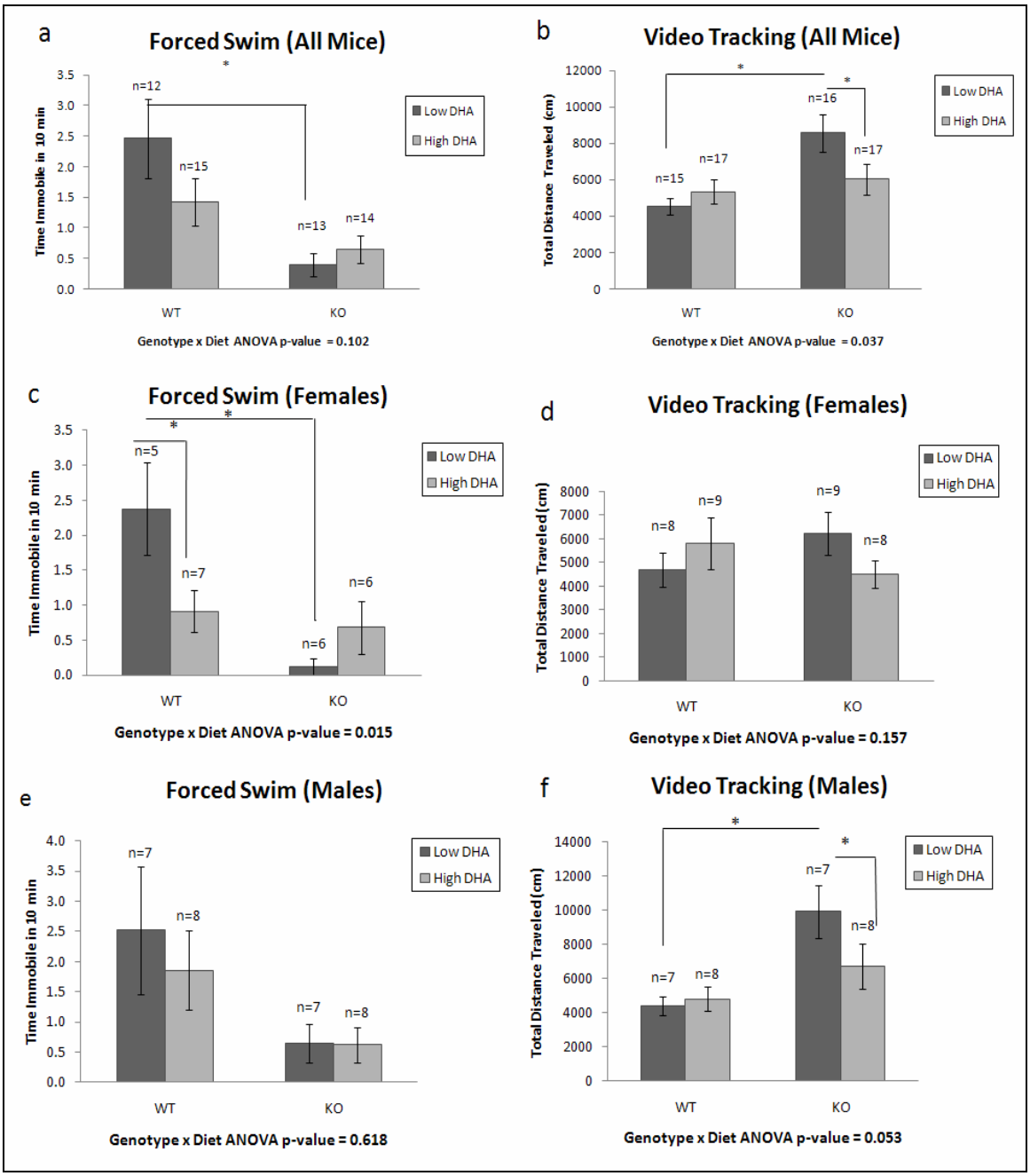


Figure 5. Effects of DHA on ST DBP KO and ST WT mice behavior. DBP mice on a diet supplemented with low or high DHA were subjected to a chronic stress paradigm consisting of isolation (single housing) for 4 weeks, with an acute stressor (behavioral challenge tests) at day 21. Panels a, c, and e show forced swim test results. After 21 days of the stress paradigm, the forced swim test was used to measure immobility during a 10

min period. Panels b, d and f show open-field video tracking results. After the 28 day stress paradigm, the open-field test was used to measure total distance traveled (in centimeters) during a 30 min period. Two-factor ANOVA testing Genotype and Diet was employed. * indicates t-test p-value ≤ 0.05

DHA-Induced Changes in Gene Expression Patterns

For our gene expression studies based on microarray data, the CFG approach was used (Figure 6) and the scoring process was described in the Material and Methods section. With a minimum CFG cut-off score of 2.0 points, we were able to generate a list of 72 genes for females and 40 genes for males that were changed with a p-value threshold for change of $p<0.0025$ in at least two out of three microarray experiments comparing ST DBP KO mice on a high verses low DHA diet (Tables 1 and 2). Among the genes changed by DHA treatment in female mice were CD44, MBP, DRD2, ERBB3, GSN, MAL, MAG, NEFH, ESR1, SYT2, CNP, MOG, CLDN11, QDPR, PTN, CRYAB, KIF5C, CSRP1, ST18, APOD, SYN2, GPR37, PRDX2, MOBP, PLP1, and PDE8A. These genes were changed in the opposite direction in the PFC of the ST KO mice on the high DHA diet as compared to the postmortem brain or blood of humans with bipolar disorder, alcoholism, anxiety disorders, or major depression (Table 1). According to CFG of microarray data for male ST DBP KO mice on the high verses low DHA diet, SFPQ, ATRX, ARF3, NDUFAB, FOS, ADAR, MAX, TXNDC13, UBE2D2, COL16A1, and TIMM9 were changed in the opposite direction in the PFC of the male ST KO mice on the high DHA diet as compared to the postmortem brain or blood of humans with bipolar disorder, alcoholism, anxiety disorders, or major depression (Table 2).

Based on the CFG tables, pyramids were made providing the top candidate genes (3.0 points and higher) changed in female and male ST KO mice on the high verses low DHA diet, with the genes receiving the highest CFG score placed at the top (Figure 7, A and B). The genes are also designated by the line of evidence for which they were reported. Based on these findings, genes serving as potential biomarkers for monitoring

of DHA treatment in ST DBP KO mice include CD44, EFHD1, and TFRC, as they were changed in the same direction in the brain and blood of ST DBP KO mice.

Overlap with Previous Findings

Previous microarray and CFG data on ST DBP KO mice PFC brain regions compared to WT mice PFC brain regions revealed a list of top candidate genes for bipolar disorder and stress response [31]. In that study, ADP-ribosylation factor 3 (Arf3) was decreased in the PFC of male ST KO mice compared to WT control. In the present study, Arf3 was increased in the PFC of male ST KO mice on a high DHA diet compared to their male counterparts on a low DHA diet, showing the potential role of omega-3 fatty acids in the reversal of the deficit in this gene in the brain. Interestingly, the expression of the genes MBP, DRD2, MAL, CNP, MOG, CLDN11, CSRP1, and PLP1 changed in the opposite direction in female ST KO mice on the high DHA diet compared to the male ST KO mice on a control diet from the previous DBP KO study (Table 2).

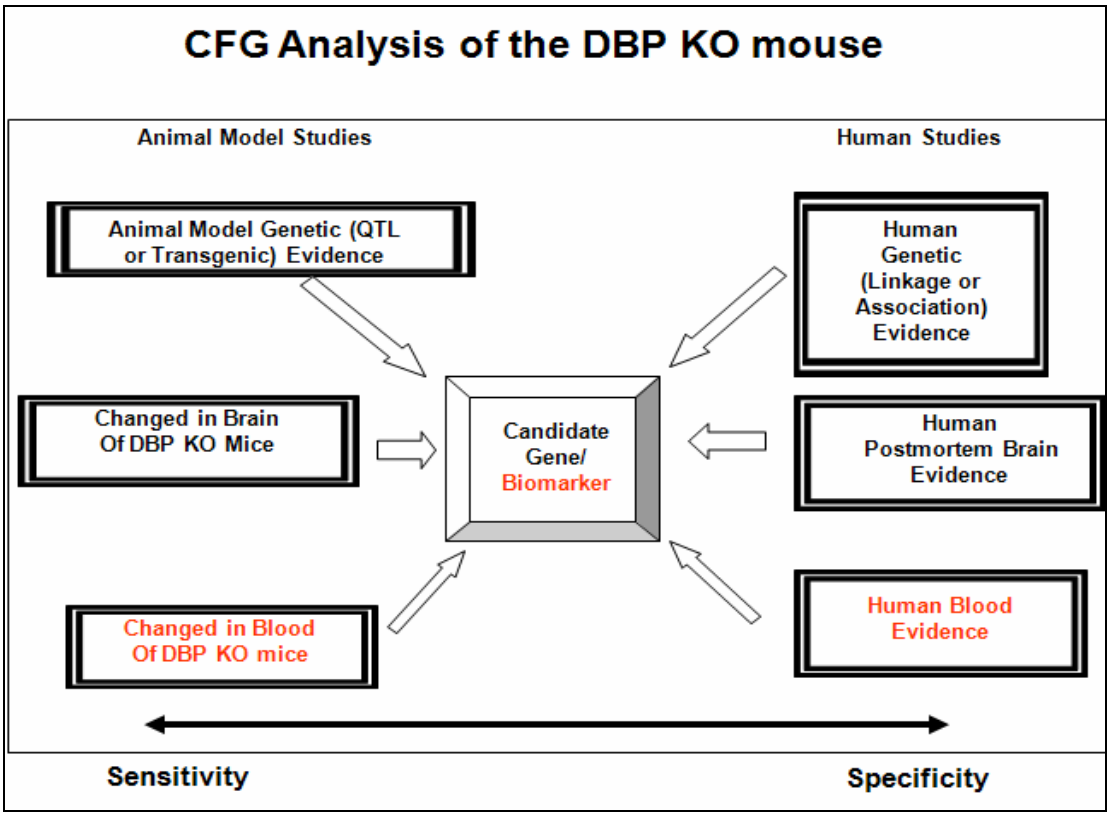


Figure 6. Expanded Convergent Functional Genomics (CFG) analysis. Using the integration of multiple animal model and human lines of evidence, genes were scored that significantly changed in two out of three experiments in the brain or blood of ST DBP KO mice on the high DHA diet compared to ST DBP KO mice on the low DHA diet. Genes changed in the PFC received 1 point; genes changed in the blood received an additional 1 point. If the brain and blood direction of change was concordant, the gene received an additional 2 points. The other four lines of evidence received a maximum of 1 point each (for human genetic data: 0.5 points if it was linkage, 1 point if it was association; for mouse genetic data: 0.5 points if it was QTL, 1 point if it was transgenic). Thus, the maximum possible CFG score for each gene was 8 points.

Table 1. Top ST DBP KO gene expression changes in female mice on high DHA vs. low DHA diet**.** Top candidate genes for which there were changes in prefrontal cortex (PFC) and/or blood are shown with a CFG score of 2 points and higher. I – Increased in expression, D – decreased in expression, BP – bipolar, MDD – major depressive disorder, PTSD – post traumatic stress disorder.

Gene Symbol/ Name	Mouse PFC	Mouse Blood	Mouse Brain-Blood Concordance	Human Genetic Evidence (Linkage or Association)	Human Postmortem Brain Evidence	Human Blood Evidence	Mouse Genetic Evidence (QTL, TG)	CFG Score
CD44 CD44 antigen	D	D	Yes	11p13 BP[68-69]	D (Alcohol)[70]	I (BP)[71]	Abnormal Emotion/Affect Behavior	7.0
CLIC4 chloride intracellular channel 4 (mitochondrial)	I	D	No	1p36.11 BP[72]	I (PTSD)[73] D (Alcohol)[74]	I (BP)[71]	(Transgenic) Abnormal Emotion/Affect Behavior	5.5
DRD2 dopamine receptor 2	I			11q23.2 BP[75] (Association) PTSD[76] Alcohol and Drug Dependence[77] Alcohol Addiction[78] Alcohol Dependence[79] Alcohol[80-83]	I (BP)[84] D (Depression)[85] D (Alcohol)[81]	D (Delusions) [86]	Abnormal Emotion/Affect Behavior Abnormal Circadian Rhythm (Transgenic) Abnormal Emotion/Affect Behavior	5.0
MBP myelin basic protein	I	D	No	18q23 (Association) Substance Dependence[87] BP[88-93]	D (BP)[94] D (Alcohol)[70, 95] I (Male BP)[96] D (Female BP)[96]	I (Mood)[97]		5.0
CDKN1C cyclin-dependent kinase inhibitor 1C (P57)	D			11p15.4 (Association) BP[93]	D (MDD)[98]	D (Chronic Stress)[99]	Abnormal Emotion/Affect Behavior Addiction/Drug Abuse	4.5

Gene Symbol/ Name	Mouse PFC	Mouse Blood	Mouse Brain-Blood Concordance	Human Genetic Evidence (Linkage or Association)	Human Postmortem Brain Evidence	Human Blood Evidence	Mouse Genetic Evidence (QTL, TG)	CFG Score
NEFH neurofilament, heavy polypeptide	I			22q12.2 BP[100-101]	D (Alcohol)[102] D (BP)[103] I (Alcohol)[104]	I (Hallucinations) [86]	Addiction/Drug Abuse Abnormal Emotion/Affect Behavior (Transgenic) Abnormal Emotion/Affect Behavior	4.5
PRKAR2B protein kinase, cAMP dependent regulatory, type II beta	I	D	No	7q22.3 Panic Disorder[105] Alcohol[106-107]	I (BP)[108] D (PTSD)[73]		(Transgenic) Addiction/Drug Abuse	4.5
CAST calpastatin	I			5q15 Alcohol[109] (Association) Alcohol[110]	D (Alcohol)[74]		(Transgenic) Abnormal Circadian Rhythm	4.0
EFHD1 EF hand domain containing 1	I	I	Yes	2q37.1				4.0
ERBB3 v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian)	I			12q13.2 Panic Disorder[111-112]	D (MDD)[98] D (BP)[94]	D (Mood)[97]	Abnormal Sleep Pattern Abnormal Emotion/Affect Behavior	4.0
GSN gelsolin	I	D	No	9q33.2 BP[69, 113] Alcohol[114]	D (BP)[108, 115]		Addiction/Drug Abuse Abnormal Emotion/Affect Behavior Abnormal Circadian Rhythm	4.0

Gene Symbol/ Name	Mouse PFC	Mouse Blood	Mouse Brain-Blood Concordance	Human Genetic Evidence (Linkage or Association)	Human Postmortem Brain Evidence	Human Blood Evidence	Mouse Genetic Evidence (QTL, TG)	CFG Score
MAG myelin-associated glycoprotein	I			19q13.12 MDD[116]	D (MDD)[98, 117] D (Alcohol)[70] I (Alcohol)[102, 104]	D (Mood)[97]	Abnormal Emotion/Affect Behavior Abnormal Sleep Pattern Addiction/Drug Abuse Abnormal Circadian Rhythm	4.0
PPP1R1B protein phosphatase 1, regulatory (inhibitor) subunit 1B	I			17q12 Alcohol[118] (Association) Alcohol[119]	D (BP)[120]		(Transgenic) Addiction/Drug Abuse	4.0
SEPT8 septin 8	I			5q23.3 MDD[116]	D (Alcohol)[70]	D (Mood)[97]	Abnormal Emotion/Affect Behavior Addiction/Drug Abuse Abnormal Circadian Rhythm	4.0
ESR1 estrogen receptor 1 (alpha)	D			6q25.1 (Association) Childhood Onset Mood Disorder[121] Alcohol[110]	I (Depression)[122]		Addiction/Drug Abuse Abnormal Emotion/Affect Behavior	3.5
MAL myelin and lymphocyte protein, T-cell differentiation protein	I			2q11.1 Alcohol[106]	D (MDD)[98] D (Alcohol)[70] I (Alcohol)[102]	I (BP)[123] D (BP)[71]		3.5
ALDH1A1 aldehyde dehydrogenase family 1, subfamily A1	I			9q21.13 Alcohol[124-125] BP[69, 126-127] Alcohol[127]	I (BP)[128]			3.0

Gene Symbol/ Name	Mouse PFC	Mouse Blood	Mouse Brain-Blood Concordance	Human Genetic Evidence (Linkage or Association)	Human Postmortem Brain Evidence	Human Blood Evidence	Mouse Genetic Evidence (QTL, TG)	CFG Score
APOD apolipoprotein D	I			3q29 BP[129]	D (MDD)[98] D (Alcohol)[70, 130] I (Alcohol)[95, 104] I (BP)[131]		Abnormal Emotion/Affect Behavior	3.0
CARHSP1 calcium regulated heat stable protein 1	I			16p13.2 Alcohol[132] BP[133]	D (Alcohol)[74]		Abnormal Emotion/Affect Behavior Abnormal Circadian Rhythm	3.0
CLDN11 claudin 11	I			3q26.2 BP[69, 72]	D (BP)[94]		Abnormal Sleep Pattern	3.0
CNP 2',3'-cyclic nucleotide 3' phosphor-diesterase	I			17q21.2 Alcohol[134]	D (MDD)[98] D (Alcohol)[70, 74, 135]		Abnormal Sleep Pattern Abnormal Emotion/Affect Behavior Addiction/Drug Abuse	3.0
CRYAB crystallin, alpha B	I			11q23.1 Alcohol[80]	D (Stress Disorder)[136] I (Alcohol)[102, 104] D (PTSD)[73]		Abnormal Emotion/Affect Behavior Abnormal Circadian Rhythm	3.0
CTGF connective tissue growth factor	I	D	No	6q23.2 BP[137-138]			Addiction/Drug Abuse Abnormal Emotion/Affect Behavior Abnormal Circadian Rhythm	3.0
EVI2A ecotropic viral integration site 2a	I	D	No	17q11.2 Alcohol[109] BP[69, 139]			Abnormal Emotion/Affect Behavior Abnormal Sleep Pattern Addiction/Drug Abuse	3.0

Gene Symbol/ Name	Mouse PFC	Mouse Blood	Mouse Brain-Blood Concordance	Human Genetic Evidence (Linkage or Association)	Human Postmortem Brain Evidence	Human Blood Evidence	Mouse Genetic Evidence (QTL, TG)	CFG Score
FABP7 fatty acid binding protein 7, brain	D			6q22.31 Alcohol[80] BP[140-142] (Association) Panic Disorder[143] BP[142]	D (BP)[108]			3.0
FOXO3A forkhead box O3a	I			6q21 BP[140-141, 144-146] Anxiety, Depression[147] Alcohol[80]	I (BP)[103]		Abnormal Circadian Rhythm Abnormal Emotion/Affect Behavior Addiction/Drug Abuse	3.0
GPR37 G protein-coupled receptor 37	I			7q31.33 BP[148]	D (MDD)[98] D (Alcohol)[70]		Abnormal Emotion/Affect Behavior	3.0
GRP gastrin releasing peptide	D			18q21.32 BP[92, 140, 149] (Association) Panic Disorder[143, 150]			Abnormal Emotion/Affect Behavior (Transgenic) Addiction/Drug Abuse	3.0
KIF5C kinesin family member 5C	I			2q23.1 BP[72, 105] Alcohol[124]		D (Mood)[97] I (Delusions)[86]	Abnormal Circadian Rhythm Abnormal Emotion/Affect Behavior	3.0
MOG myelin oligodendrocyte glycoprotein	I			6p22.1 BP[151]	D (BP)[94] D (MDD)[98] D (Alcohol)[70]		Abnormal Emotion/Affect Behavior	3.0
PRDX2 peroxiredoxin 2	D			19p13.13 MDD[116]	D (BP)[128] I (Alcohol)[152]		Abnormal Circadian Rhythm Abnormal Emotion/Affect Behavior Addiction/Drug Abuse	3.0

Gene Symbol/ Name	Mouse PFC	Mouse Blood	Mouse Brain-Blood Concordance	Human Genetic Evidence (Linkage or Association)	Human Postmortem Brain Evidence	Human Blood Evidence	Mouse Genetic Evidence (QTL, TG)	CFG Score
PTN pleiotrophin	D			7q33 BP[69]	I (Major Depression)[153] I (Alcohol)[74]		Abnormal Emotion/Affect Behavior	3.0
QDPR quinoid dihydropteridine reductase	I			4p15.31 BP[154]	D (BP)[103]		Abnormal Emotion/Affect Behavior Abnormal Circadian Rhythm	3.0
SPTBN1 spectrin, beta, non-erythrocytic 1	I	D	No	2p16.2 BP[139] Alcohol[80, 106]			Abnormal Emotion/Affect Behavior Addiction/Drug Abuse	3.0
SYT2 synaptotagmin II	I			1q32.1 Panic Disorder[112] Alcohol[80]	D (BP)[103]		Abnormal Emotion/Affect Behavior	3.0
CAMK4 calcium/calmod-ulin-dependent protein kinase IV	I			5q22.1 Alcohol[109]			Abnormal Emotion/Affect Behavior Addiction/Drug Abuse (Transgenic) Addiction/Drug Abuse	2.5
CPM carboxy-peptidase M	I			12q15 BP[155]		I (Mood)[97]		2.5
CSRP1 cysteine and glycine-rich protein 1	I			1q32.1 Panic Disorder[112] Alcohol[80]	D (Alcohol)[70, 156]			2.5
DRD1A dopamine receptor D1A	I			5q35.2 BP[157] Panic Disorder[158]			Addiction/Drug Abuse Abnormal Emotion/Affect Behavior (Transgenic) Addiction/Drug Abuse	2.5

Gene Symbol/ Name	Mouse PFC	Mouse Blood	Mouse Brain-Blood Concordance	Human Genetic Evidence (Linkage or Association)	Human Postmortem Brain Evidence	Human Blood Evidence	Mouse Genetic Evidence (QTL, TG)	CFG Score
GAB1 growth factor receptor bound protein 2-associated protein 1	I			4q31.21 BP[154, 159]	D (Alcohol)[74]			2.5
GPR6 G protein-coupled receptor 6	I			6q21 (Association) BP[93, 141, 144-146] Anxiety, Depression[147] Alcohol[80]			Abnormal Circadian Rhythm Abnormal Emotion/Affect Behavior Addiction/Drug Abuse	2.5
LITAF LPS-induced TN factor	I	D	No	BP[105, 133]				2.5
MOBP myelin-associated oligodendrocytic basic protein	I			3p22.2	D (BP)[94] D (MDD)[98] D (Alcohol)[70] I (Alcohol)[102, 104]		Abnormal Emotion/Affect Behavior Abnormal Circadian Rhythm	2.5
NDRG1 N-myc downstream regulated gene 1	I	D	No	BP[69, 146, 149]				2.5
PTTG1 pituitary tumor-transforming 1	D			5q33.3 BP[36, 160]		I (Postpartum Depression) [161]		2.5
RASGRP2 RAS, guanyl releasing protein 2	I	D	No	BP[69, 162]				2.5

Gene Symbol/ Name	Mouse PFC	Mouse Blood	Mouse Brain-Blood Concordance	Human Genetic Evidence (Linkage or Association)	Human Postmortem Brain Evidence	Human Blood Evidence	Mouse Genetic Evidence (QTL, TG)	CFG Score
RGS9 regulator of G-protein signaling 9	I			17q24.1		D (Mood)[97]	Abnormal Sleep Pattern Abnormal Emotion/Affect Behavior Addiction/Drug Abuse	2.5
ST18 suppression of tumorigenicity 18	I			8q11.23 BP[92, 140]	D (Alcohol)[130]			2.5
SYN2 synapsin II	D			3p25	I (Major Depression Suicide Completer)[163-164] D (Alcohol)[104] D (BP)[165]		Abnormal Circadian Rhythm Abnormal Emotion/Affect Behavior Abnormal Sleep Pattern	2.5
CACNA2D1 calcium channel, voltage-dependent, alpha2/delta subunit 1	D			7q21.11 Panic Disorder[105] Alcoholism[166] BP[139, 145]			Abnormal Emotion/Affect Behavior	2.0
COL11A1 collagen, type XI, alpha 1	I			1p21.1 Alcohol[124, 167-168] Depression, Alcohol[169]			Addiction/Drug Abuse Abnormal Sleep Pattern Abnormal Emotion/Affect Behavior	2.0
CPLX3 complexin 3	I			15q24.1		I (Mood)[97]		2.0
ELOVL7 ELOVL family member 7, elongation of long chain fatty acids (yeast)	I			5q12.1	D (Alcohol)[74]			2.0

Gene Symbol/ Name	Mouse PFC	Mouse Blood	Mouse Brain-Blood Concordance	Human Genetic Evidence (Linkage or Association)	Human Postmortem Brain Evidence	Human Blood Evidence	Mouse Genetic Evidence (QTL, TG)	CFG Score
EOMES eomesodermin homolog (Xenopus laevis)	D			3p24.1			Abnormal Emotion/Affect Behavior Abnormal Circadian Rhythm (Transgenic) Abnormal Emotion/Affect Behavior	2.0
FA2H fatty acid 2-hydroxylase	I			16q22.3		I (Mood)[97] D (Delusions)[86]		2.0
HAPLN2 hyaluronan and proteoglycan link protein 2	I			1q23.1 Alcoholism[167] BP[162]			Addiction/Drug Abuse Abnormal Sleep Pattern Abnormal Emotion/Affect Behavior	2.0
LASS2 LAG1 homolog, ceramide synthase 2	I			1q21.2	D (Alcohol)[74]			2.0
LGI2 leucine-rich repeat LGI family, member 2	I			4p15.2 (Association) BP, Substance Dependence[87]				2.0
MGP matrix Gla protein	I			12p12.3		D (Alcohol)[170]		2.0
PDE10A Phospho-diesterase 10A	I			6q27 (Association) MDD[171]				2.0
PDE8A phosphor-diesterase 8A	I			15q25.3	D (MDD)[98]			2.0

Gene Symbol/ Name	Mouse PFC	Mouse Blood	Mouse Brain-Blood Concordance	Human Genetic Evidence (Linkage or Association)	Human Postmortem Brain Evidence	Human Blood Evidence	Mouse Genetic Evidence (QTL, TG)	CFG Score
PENK1 preproen-kephalin 1	I			8q12.1			Addiction/Drug Abuse (Transgenic) Addiction/Drug Abuse	2.0
PLEKHH1 pleckstrin homology domain containing, family H (with MyTH4 domain) member 1	I			14q24.1	D (Alcohol)[74]			2.0
PLP1 proteolipid protein (myelin) 1	I			Xq22.2	D (BP)[94] D (MDD)[98, 117] D (Alcohol)[74] I (Alcohol)[104]			2.0
SCN7A sodium channel, voltage-gated, type VII, alpha	D			2q24.3 BP[105] Alcohol[124]			Abnormal Circadian Rhythm Abnormal Emotion/Affect Behavior	2.0
SDC4 syndecan 4	D			20q13.12 Alcohol[109] BP[172]			Addiction/Drug Abuse Abnormal Emotion/Affect Behavior	2.0
SESN3 sestrin 3	D			11q21	I (Alcohol)[74]			2.0
SLC44A1 solute carrier family 44, member 1	I	D	No					2.0
TNNT2 troponin T2, cardiac	D			1q32 Panic Disorder[112] Alcohol[80]			Abnormal Emotion/Affect Behavior	2.0

Gene Symbol/ Name	Mouse PFC	Mouse Blood	Mouse Brain-Blood Concordance	Human Genetic Evidence (Linkage or Association)	Human Postmortem Brain Evidence	Human Blood Evidence	Mouse Genetic Evidence (QTL, TG)	CFG Score
TPBG trophoblast glycoprotein	I			6q14.1		I (Valproate Treated-Human NBFL Cells)[103]		2.0
TSPAN2 tetraspanin 2	I	D	No					2.0
TTYH2 tweety homolog 2 (Drosophila)	I			17q25.1	D (Alcohol)[74]			2.0

Table 2. Top ST DBP KO gene expression changes in male mice on high DHA vs. low DHA diet. Top candidate genes for which there were changes in prefrontal cortex (PFC) and/or blood are shown with a CFG score of 2 points and higher. I – Increased in expression, D – decreased in expression, BP – bipolar, MDD – major depressive disorder, PTSD – post traumatic stress disorder.

Gene Symbol/ Name	Mouse PFC	Mouse Blood	Mouse Brain-Blood Concordance	Human Genetic Evidence (Linkage or Association)	Human Postmortem Brain Evidence	Human Blood Evidence	Mouse Genetic Evidence (QTL, TG)	CFG Score
TFRC transferrin receptor	I	I	Yes	3q29 BP[92, 173-174] Alcohol[175]			Abnormal Emotion/Affect Behavior	5.0
FOS FBJ osteosarcoma oncogene	D			14q24.3 BP [69] Alcohol[109] Simple Phobia[176]	I (BP)[177]	I (PTSD)[178] (Stress)[179-181]	Abnormal Sleep Pattern Abnormal Emotion/Affect Behavior (Transgenic) Abnormal Emotion/Affect Behavior, Abnormal Sleep Pattern	4.5
PAFAH1B1/LIS1 platelet-activating factor acetylhydrolase, isoform 1b, beta1 subunit	D			17p13.3 (Association) BP[182]	I (Alcohol)[70]	D (Chronic Stress)[99] D (Mood)[97]	Abnormal Emotion/Affect Behavior Abnormal Sleep Pattern Addiction/Drug Abuse	4.5
SFPQ splicing factor proline/glutamine rich (polypyrimidine tract binding protein associated)	I	D	No	1p34.3	I (BP)[108] I (MDD)[153]	I (Social Isolation)[183]	Abnormal Emotion/Affect Behavior Addiction/Drug Abuse	4.5

Gene Symbol/ Name	Mouse PFC	Mouse Blood	Mouse Brain-Blood Concordance	Human Genetic Evidence (Linkage or Association)	Human Postmortem Brain Evidence	Human Blood Evidence	Mouse Genetic Evidence (QTL, TG)	CFG Score
NDUFAB1 NADH dehydrogenase (ubiqui) 1, alpha/beta subcomplex, 1	I			16p12.1 (Association) BP[92, 105, 184]	D (BP)[185]	D (Chronic Stress)[99]		4.0
ARF3 ADP-ribosylation factor 3	I			12q13.12 Panic Disorder[111-112]	D (Alcohol)[70]	D (Chronic Stress)[99]		3.5
PTTG1 pituitary tumor-transforming 1	D	I	No	5q33.3 BP[36, 160]		I (Postpartum Depression PBMC)[161]		3.5
BNIP3L BCL2/adenovirus E1B interacting protein 3-like	D	I	No	8p21.2 BP[72, 140, 186]			Abnormal Emotion/Affect Behavior	3.0
CNR1 cannabinoid receptor 1 (brain)	I			6q15 (Association) PTSD[187]			Abnormal Emotion/Affect Behavior Addiction/Drug Abuse (Transgenic) Addiction/Drug Abuse, Abnormal Emotion/Affect Behavior	3.0
DSC2 desmocollin 2	D			18q12.1 BP[188]	D (BP)[189] I (MDD)[189] D (Alcohol)[70]		Addiction/Drug Abuse Abnormal Emotion/Affect Behavior	3.0
HSPA4 heat shock protein 4	D			5q31.1 MDD[116]		D (Stress) [179, 181]	Addiction/Drug Abuse Abnormal Emotion/Affect Behavior Abnormal Circadian Rhythm	3.0
MAX MYC associated factor X	D			14q23.3 BP[72] Alcohol[109]		I (BP)[71]	Abnormal Sleep Pattern Abnormal Emotion/Affect Behavior	3.0

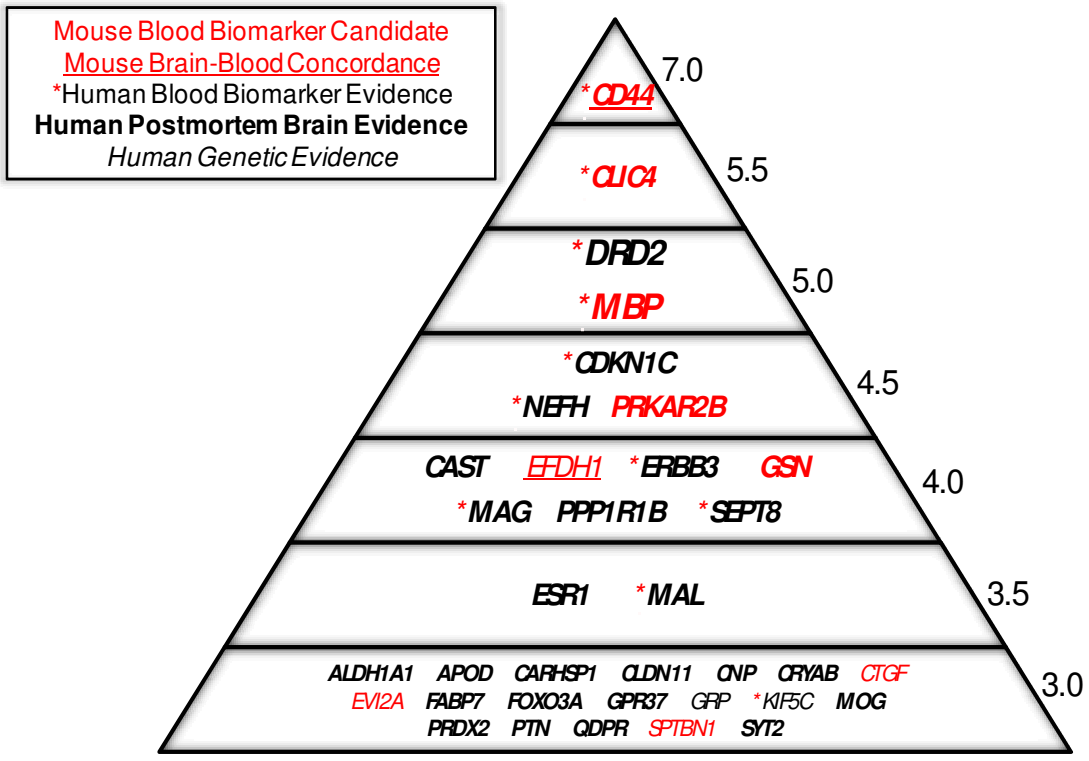
Gene Symbol/ Name	Mouse PFC	Mouse Blood	Mouse Brain-Blood Concordance	Human Genetic Evidence (Linkage or Association)	Human Postmortem Brain Evidence	Human Blood Evidence	Mouse Genetic Evidence (QTL, TG)	CFG Score
MTDH Metadherin	D			8q22.1 BP[72, 190]		D (Chronic Stress)[99]	Abnormal Emotion/Affect Behavior	3.0
SLC2A3 solute carrier family 2 (facilitated glucose transporter), member 3	I			12p13.31 Alcohol[109]		I (Chronic Stress)[99]	Abnormal Circadian Rhythm Abnormal Emotion/Affect Behavior Abnormal Sleep Pattern Addiction/Drug Abuse	3.0
TMX4 thioredoxin-related transmembrane protein 4	I			20p12.3 BP[133, 172, 186, 191-192]		D (Chronic Stress)[99]	Abnormal Emotion/Affect Behavior Addiction/Drug Abuse Abnormal Circadian Rhythm	3.0
ADAR adenosine deaminase, RNA-specific	I			1q21.3 Alcohol[167]		D (Chronic Stress)[99]		2.5
ATRX alpha thalassemia/mental retardation syndrome X-linked homolog (human)	I			Xq21.1	D (Alcohol)[156]		Abnormal Emotion/Affect Behavior	2.5
GPRC5B G protein-coupled receptor, family C, group 5, member B	D			16p12.3 BP[92, 105]	D (MDD)[98]			2.5
LUC7L2 LUC7-like 2 (S. cerevisiae)	I	D	No	7q34 Unipolar[129] BP[69]				2.5
PNN Pinin	I			14q21.1 Alcohol[109]	I (Alcohol)[74]			2.5

Gene Symbol/ Name	Mouse PFC	Mouse Blood	Mouse Brain-Blood Concordance	Human Genetic Evidence (Linkage or Association)	Human Postmortem Brain Evidence	Human Blood Evidence	Mouse Genetic Evidence (QTL, TG)	CFG Score
RALY hnRNP-associated with lethal yellow	D			20q11.22 BP[69, 172]			Abnormal Emotion/Affect Behavior	2.5
SFRS5 splicing factor, arginine/serine-rich 5 (SRp40, HRS)	I			14q24.1 Simple Phobia[176] Alcohol[109] BP[69]	I (Stress)[136]			2.5
TEF thyrotroph embryonic factor	I			22q13.2 (Association) Unipolar[193] BP[91, 100] Panic Disorder[158]			Abnormal Emotion/Affect Behavior	2.5
UBE2D2 ubiquitin-conjugating enzyme E2D 2	MI			5q31.2 BP[139]	D (Stress)[136]			2.5
VAMP2 vesicle-associated membrane protein 2	I			17p13.1 Alcohol[109]	I (Mood Disorders)[194]			2.5
YWHAG tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma polypeptide	I			7q11.23 Alcoholism[166]	D (Alcohol)[135]			2.5
CAP1 CAP, adenylate cyclase-associated protein 1 (yeast)	D			1p34.2	D (BP)[115]			2.0
COL16A1 collagen, type XVI, alpha 1	I			1p35.2	D (BP)[195]			2.0

Gene Symbol/ Name	Mouse PFC	Mouse Blood	Mouse Brain-Blood Concordance	Human Genetic Evidence (Linkage or Association)	Human Postmortem Brain Evidence	Human Blood Evidence	Mouse Genetic Evidence (QTL, TG)	CFG Score
DCT dopachrome tautomerase	I			13q32.1 BP[100] Panic Disorder[158]			Abnormal Emotion/Affect Behavior	2.0
FMN2 formin 2	I			1q43 Panic Disorder[111, 196] Alcohol[118]			Addiction/Drug Abuse Abnormal Emotion/Affect Behavior	2.0
FSCN1 fascin homolog 1, actin bundling protein (Strongylocentrotus purpuratus)	I			7p22.1 Anxiety, Depression[147] Alcohol[109]			Abnormal Circadian Rhythm	2.0
GNG4 guanine nucleotide binding protein (G protein), gamma 4	D			1q42.3 BP[126, 129] Panic Disorder[158]			Abnormal Sleep Pattern Abnormal Emotion/Affect Behavior	2.0
HERC5 hect domain and RLD 5	D			4q22.1		D (Chronic Stress)[99]		2.0
IGH-6 immunoglobulin heavy chain 6 (heavy chain of IgM)	D			14q32.33 Anxiety[197]			Abnormal Emotion/Affect Behavior Abnormal Circadian Rhythm	2.0
PBX3 pre B-cell leukemia transcription factor 3	D			9q33.3 Alcoholism[114] BP[69, 113]			Addiction/Drug Abuse Abnormal Emotion/Affect Behavior	2.0
PRMT1 protein arginine N-methyltransferase 1	I			19q13.33 BP[69]			Addiction/Drug Abuse Abnormal Circadian Rhythm Abnormal Emotion/Affect Behavior	2.0

Gene Symbol/ Name	Mouse PFC	Mouse Blood	Mouse Brain-Blood Concordance	Human Genetic Evidence (Linkage or Association)	Human Postmortem Brain Evidence	Human Blood Evidence	Mouse Genetic Evidence (QTL, TG)	CFG Score
RGS5 regulator of G-protein signaling 5	I			1q23.3 Alcohol[167] BP[162]			Addiction/Drug Abuse Abnormal Emotion/Affect Behavior	2.0
S100A5 S100 calcium binding protein A5	D			1q21.3 Alcohol[167]			Abnormal Emotion/Affect Behavior Abnormal Sleep Pattern	2.0
SEMA5A sema domain, seven thrombospondin repeats (type 1 and type 1-like), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 5A	D			5p15.31 Alcohol[124, 198] BP[69] (Association) BP, Substance Dependence[87]				2.0
TIMM9 translocase of inner mitochondrial membrane 9 homolog (yeast)	I			14q23.1	D (Stress)[136]			2.0

A.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Human Genetic Evidence
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*DRD2
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*MBP
*CDKN1C
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*NEFH PRKAR2B
CAST EFDH1 *ERBB3 GSN
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ESR1 *MAL
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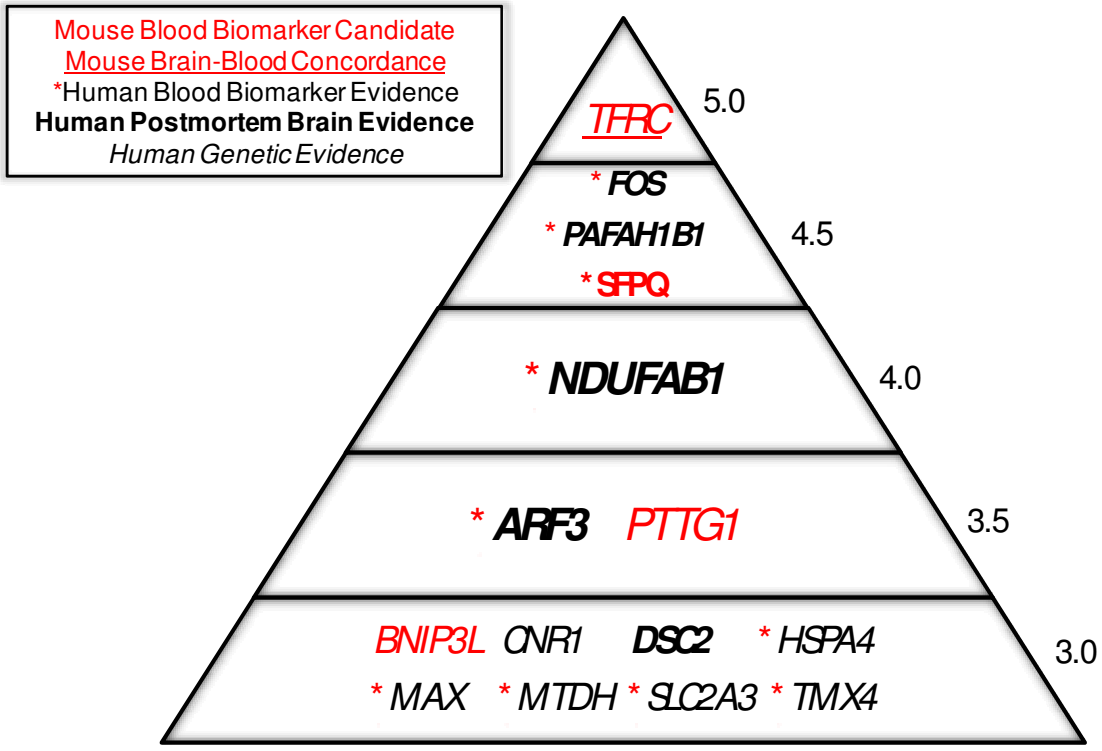
B.


Mouse Blood Biomarker Candidate
Mouse Brain-Blood Concordance
*Human Blood Biomarker Evidence
Human Postmortem Brain Evidence
Human Genetic Evidence
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TFRC
*FOS
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4.5
*SFPQ
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*ARF3 PTTG1
3.5
BNIP3L CNR1 DSC2 *HSPA4
*MAX *MTDH *SLC2A3 *TMX4
3.0

Figure 7. Top candidate gene changes in ST DBP KO mice on high vs. low DHA diet. A. Female mice; B. Male mice.

Ingenuity

To discover the molecular networks, biological functions and canonical pathways of the top candidate genes for therapy with DHA, Ingenuity Software was employed (Table 3, A and B). The list of genes from Tables 1 and 2 were separately evaluated using the software. Of particular interest, included in the molecular networks involving the top changed genes for female ST DBP KO mice were neurological disease and development of the nervous system. For top biological functions, psychological disease was associated with 29 of the genes (called molecules in Ingenuity Software) and neurological disease was connected to 48 of the genes. In the category of molecular and cellular functions, more than 30 of the genes on the female list were associated with cell death, cellular development, or cell growth and proliferation. Looking further at the table, top physiological system functions include nervous system development and behavior. Finally the top canonical pathway for the top genes changed in female ST DBP KO mice on the high DHA diet compared to their counterparts on the low DHA diet was dopamine receptor signaling.

The Ingenuity pathways for the 40 genes from male data (Table 2) did not have as high of concordance of genes as was seen for female mice, as the numbers of genes in the "Number of Molecules" column are much lower. Nevertheless, many genes were included in the top pathways. Neurological disease (6 genes) and skeletal and muscular disorders (11 genes) were the top biological functions. In the category of molecular and cellular functions, many genes were associated with cell cycle, gene expression, cellular development, cell growth and proliferation and cell signaling. Looking further at the table, top physiological system functions include embryonic, connective tissue, and

nervous system development. Finally, the top canonical pathways for the top genes changed in male ST DBP KO mice on the high DHA diet compared to their counterparts on the low DHA diet included CCRS signaling in macrophages, ERK5 signaling and Reelin signaling in neurons.

Table 3**.** Ingenuity pathway analysis of top candidate genes. A. Analysis of the top 72 candidate genes in female ST DBP KO mice on the high DHA diet compared to female ST DBP KO mice on the low DHA diet (CFG score of 2.0 and above); B. Analysis of the top 40 candidate genes in male ST DBP KO mice on the high DHA diet compared to ST DBP KO mice on the low DHA diet (CFG score of 2.0 and above).

A.

Female Data		
Top Networks		
Associated Network Functions		**Score**
Neurological Disease, Cell Morphology, Nervous System Development and Function		45
Cell-To-Cell Signaling and Interaction, Cellular Assembly and Organization, Cell Cycle		30
Neurological Disease, Cancer, Reproductive System Disease		20
Cell Signaling, Nucleic Acid Metabolism, Small Molecule Biochemistry		15
Molecular Transport, Cardiovascular Disease, Genetic Disorder		2
Top Bio Functions		
Diseases and Disorders	**p-value**	**Number of Molecules**
Neurological Disease	3.12E-12 - 8.88E-03	48
Psychological Disorders	1.64E-10 - 5.48E-03	29
Genetic Disorder	2.28E-10 - 8.88E-03	57
Skeletal and Muscular Disorders	4.09E-09 - 8.88E-03	32
Cancer	4.45E-07 - 8.88E-03	35
Molecular and Cellular Functions		
Name	**p-value**	**Number of Molecules**
Cell Death	8.15E-08 - 8.88E-03	31
Cellular Development	5.37E-07 - 8.88E-03	32
Cellular Assembly and Organization	7.90E-07 - 8.88E-03	24
Cell Morphology	1.67E-06 - 8.88E-03	17
Cellular Growth and Proliferation	4.86E-06 - 8.88E-03	31
Physiological System Development and Function		
Name	**p-value**	**Number of Molecules**
Nervous System Development and Function	1.67E-06 - 8.88E-03	27
Behavior	6.03E-06 - 8.88E-03	13
Skeletal and Muscular System Development and Function	1.17E-05 - 8.88E-03	14
Organ Development	1.95E-05 - 8.88E-03	14
Reproductive System Development and Function	1.95E-05 - 8.88E-03	9
Top Canonical Pathways		
Name	**p-value**	**Ratio**
Dopamine Receptor Signaling	3.25E-04	4/93 (0.043)
cAMP-mediated Signaling	6.24E-04	5/161 (0.031)
G-Protein Coupled Receptor Signaling	2.07E-03	5/220 (0.023)
PXR/RXR Activation	3.88E-03	3/91 (0.033)
IGF-1 Signaling	7.97E-03	3/100 (0.03)

B.

Male Data		
Top Networks		
Associated Network Functions	**Score**	
Neurological Disease, Skeletal and Muscular Disorders, Cell Cycle	48	
Cellular Development, Cellular Growth and Proliferation, Cancer	26	
Cancer, Renal and Urological Disease, Post-Translational Modification	6	
Genetic Disorder, Skeletal and Muscular Disorders, Inflammatory Disease	2	
Top Bio Functions		
Diseases and Disorders	**p-value**	**Number of Molecules**
Neurological Disease	3.53E-05 - 4.77E-02	6
Skeletal and Muscular Disorders	3.53E-05 - 4.77E-02	11
Cancer	1.11E-04 - 4.82E-02	20
Immunological Disease	1.85E-03 - 3.90E-02	14
Reproductive System Disease	2.46E-03 - 1.23E-02	9
Molecular and Cellular Functions		
Name	**p-value**	**Number of Molecules**
Cell Cycle	8.81E-05 - 3.87E-02	9
Gene Expression	1.64E-04 - 4.82E-02	7
Cellular Development	1.83E-04 - 4.82E-02	12
Cellular Growth and Proliferation	1.83E-04 - 4.82E-02	14
Cell-to-Cell Signaling and Interaction	1.94E-04 - 4.35E-02	8
Physiological System Development and Function		
Name	**p-value**	**Number of Molecules**
Embryonic Development	8.81E-05 - 4.58E-02	12
Connective Tissue Development and Function	1.83E-04 - 4.58E-02	6
Nervous System Development and Function	1.94E-04 - 4.58E-02	8
Hair and Skin Development and Function	2.10E-04 - 4.58E-02	7
Renal and Urological System Development and Function	2.10E-04 - 2.92E-02	5
Top Canonical Pathways		
Name	**p-value**	**Ratio**
CCR5 Signaling in Macrophages	1.22E-02	2/92 (0.022)
ERK5 Signaling	1.22E-02	2/71 (0.028)
Reelin Signaling in Neurons	1.46E-02	2/78 (0.026)
IL-1 Signaling	2.11E-02	2/106 (0.019)
IGF-1 Signaling	2.15E-02	2/100 (0.02)

Alcohol consumption in ST DBP KO mice and P rats

Given the high rate of co-morbidity of alcoholism and bipolar disorder in humans, and our previous study showing increased alcohol intake in ST DBP KO compared to ST WT mice [31], alcohol intake was monitored in DBP KO mice on a high or low DHA diet. In studies using male ST DBP KO mice, mice on a diet high in DHA for 14 and 28 days drank significantly less alcohol than mice on a diet low in DHA during the same amount of time (Figure 8. (a and b) 14 days of DHA treatment, t-test p-value = 0.002. (c and d) 28 days of DHA treatment, t-test p-value = 0.04). No significant differences in water consumption were observed (data not shown), which shows mice had a preference for alcohol and were not simply drinking more fluids.

The effects of DHA on another rodent model, the alcohol-preferring (P) rat, were also tested. A dose-dependent effect was observed, where P rats on a diet high in DHA drank significantly less alcohol over a 14 day period than did P rats on a diet low in DHA (Figure 9 (a and b) t-test p-value = 0.01 comparing alcohol consumption in rats on the high verses low DHA diets).

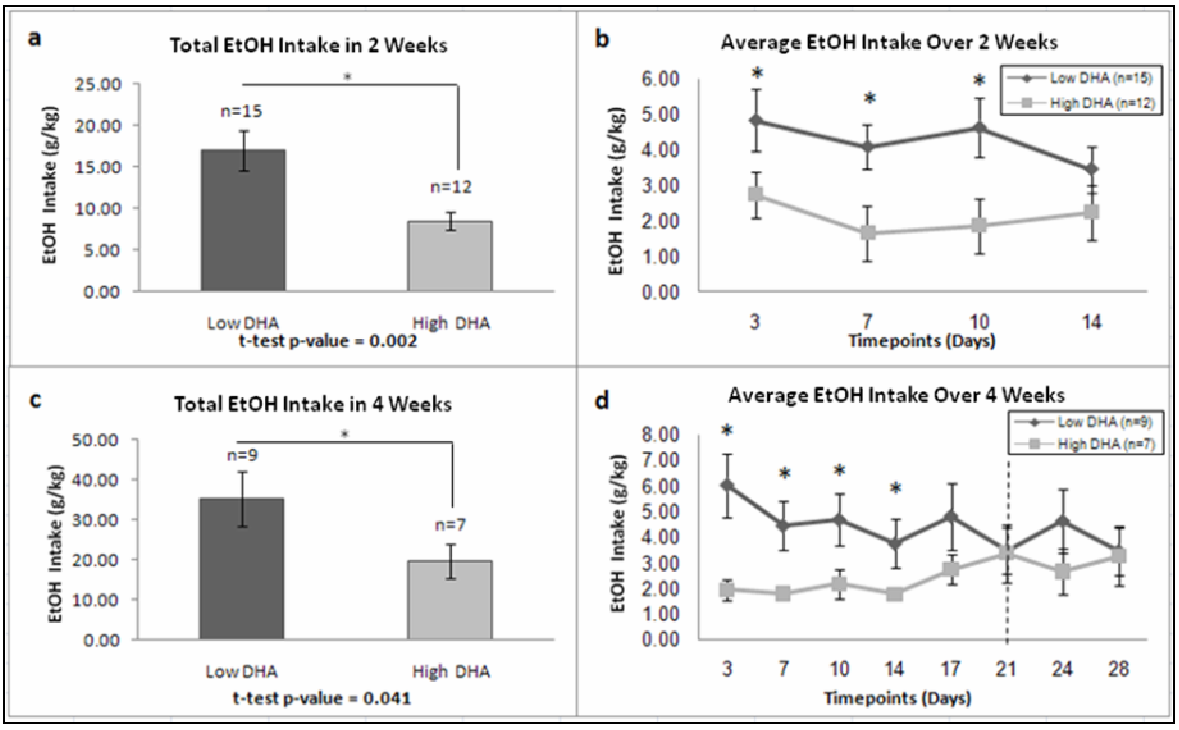

Figure 8. Effects of DHA on ethanol consumption in male ST DBP KO mice. Mice on a diet supplemented with either low or high DHA were subjected to alcohol free-choice drinking paradigm for 4 weeks. (a and b) Fluid consumption (water or 10% v/v ethanol) was monitored for a period of 2 weeks (14 days); (c and d) Fluid consumption (water or 10% v/v ethanol) was monitored for a period of 4 weeks (28 days) with an acute stressor (behavioral challenge tests represented by the dotted vertical line) on day 21. * indicates p-value ≤ 0.05.

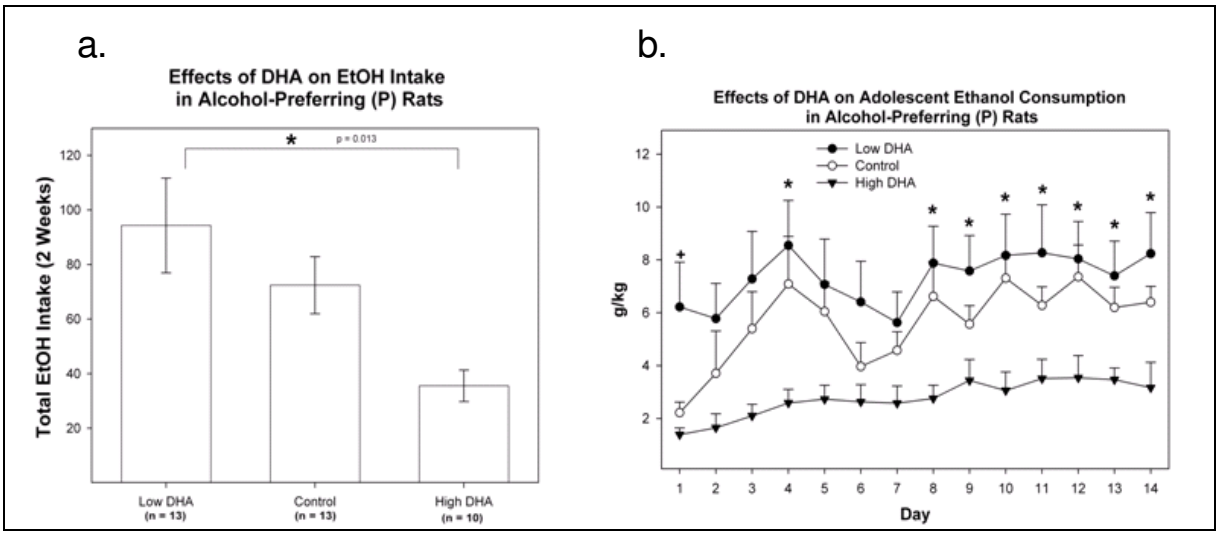

Figure 9. Effects of DHA on ethanol consumption in male alcohol-preferring (P) rats. P rats were placed on one of three diets, (1) low DHA diet, (2) control diet, or (3) high DHA diet. Rats were given continuous free-choice access in the home cage to 15% v/v ethanol and water. Ethanol intake was measured daily throughout the experiment. Fluid consumption from both bottles was monitored for a period of 2 weeks (14 days). * indicates p-value ≤ 0.05.

Discussion

The human body does not endogenously produce enough of the essential omega-3 fatty acid, DHA, and therefore it must be obtained from the diet to maintain adequate levels in phospholipids of the brain and blood [199]. This fatty acid is important for proper brain development and function, and deficits have been shown in the postmortem brains of patients with major depressive disorder and bipolar disorder [20-21]. Similar to our previous results with ST mice on a control diet [31], there was a significant difference between ST DBP KO mice and ST WT mice in the forced swim test and the open-field test when on a low DHA diet. As before, the ST DBP KO mice move significantly more than the ST WT mice. However, when mice were fed a diet high in DHA, there was no longer a significant difference in movement times (on the forced swim test for females, and on the open-field test for males). Likewise, the movement times were intermediate of those observed for the mice on the low DHA diet.

The forced swim test and open-field locomotion tracking tests have been used in studies to assess and correlate movement to mood [31, 200]. More immobility during both the forced swim and open-field tests correlates with depression, whereas heightened mobility during these tests correlates with hyperactivity and manic-like behavior. In normal humans, chronic stress can induce depression [200], whereas stress can induce mania in patients with bipolar disorder [40]. In our previous study, we showed that NST DBP KO mice present a depressed phenotype by moving significantly less than NST WT mice at baseline, but with chronic and acute stress they become more active and exhibit impulsive behavior (by increasing time spent in the center of the open field), instead of a decrease in overall locomotion as their ST WT counterparts exhibit. According to our

present results, our ST WT mice on a low DHA diet exhibit depressed behavior (low mobility in the forced swim and open-field tests), whereas ST DBP KO mice on the low DHA diet exhibit manic-like hyperactive behavior (high mobility and impulsivity), as would be expected. When given a diet high in DHA, however, the ST KO and ST WT mice no longer have polarized results, and instead have mobility times that are not significantly different and are in-between the extreme values of their counterparts on the low DHA diet, indicating the possibility of stabilized mood due to DHA in the diet.

When separating the results based on gender, significance was reached for the forced swim between the female ST WT mice on the different diets, suggesting DHA may have had an anti-depressant effect on these mice. Conversely, significance was reached for locomotion values for male ST KO mice in the open field on the different diets, suggesting DHA may have had an anti-manic effect on these mice. A trend towards anti-manic behavior was seen for female mice on the forced swim test, and with a larger cohort of animals statistical significance may be reached. Indeed, our genetic results indicated gene expression profiles congruent with anti-manic outcomes. Figure 10 depicts a hypothetical scheme derived from all of our behavioral data.

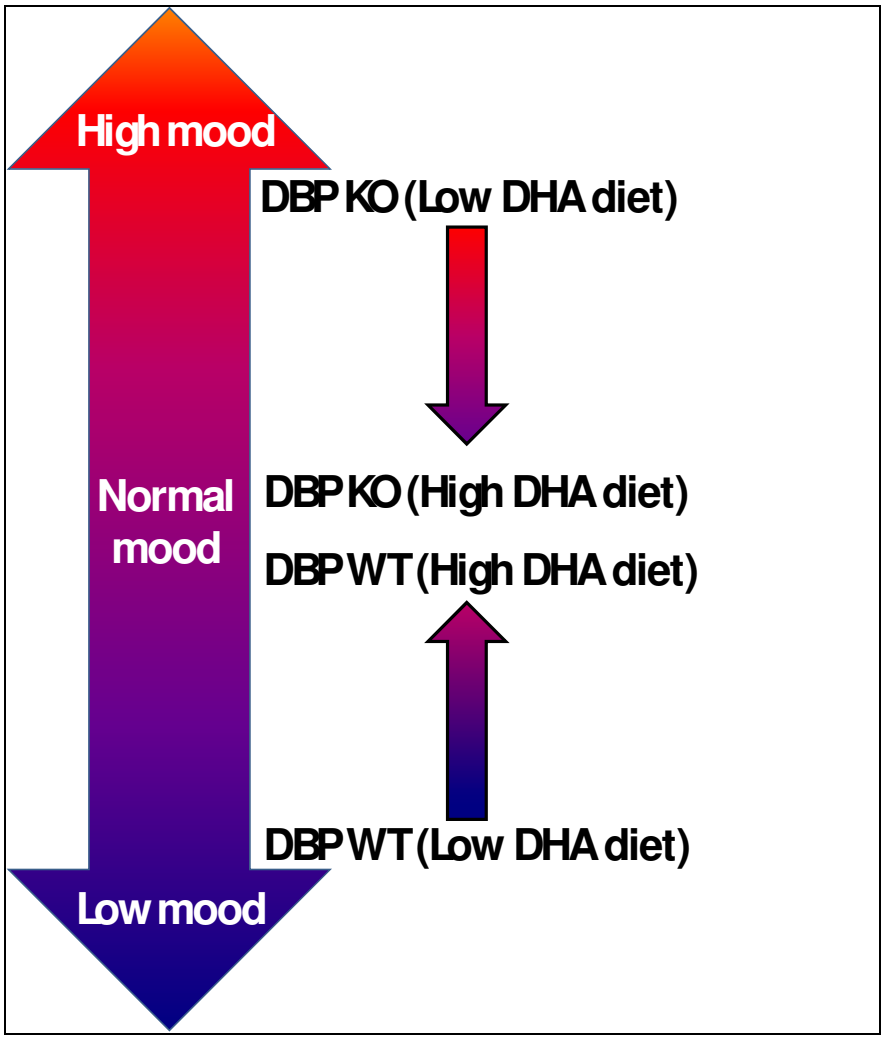

Figure 10. High DHA diet has a stabilizing effect on mood in ST DBP KO and WT mice. The high manic-like behavior seen in ST DBP KO mice on the low DHA diet and the depressed behavior of the ST WT mice on the low DHA diet are stabilized to normal behavior when given the high DHA diet.

Using the CFG approach we were able to generate a list of 40 genes for males and 72 genes for females that were changed in at least two out of three microarray replicates and which had been connected to bipolar disorder or related disorders in published literature. Many of these genes were changed in the opposite direction in the PFC of the mice on the high DHA diet compared to the postmortem PFC of bipolar patients or patients with related disorders. This shows the potential of DHA at reversing the genetic alterations seen in bipolar brains. Congruently, Ingenuity pathways for females showed that many of the genes changed by DHA have roles in neurological disease and psychological disorder, as well as behavior and neuron development. While the genes changed by DHA in males have roles in neurological disease, there are not as many disorder relevant genes as for females. Our genetic data reveals candidate genes that may have future potential in bipolar research and as targets for drug therapy.

Another goal of our genetic studies was to discover potential blood biomarkers. Blood biomarkers are genes that change in expression in the blood in concordance with the changes in the brain, and thus have potential as diagnostic tools for monitoring response to treatment [43]. We found three genes which may serve as biomarkers for monitoring response to DHA treatment. Specifically, the level of expression of CD44, EFDH1 or TFRC in the blood could be monitored during DHA supplementation, to determine subsequent genetic expression changes in the brain. If levels did not change, DHA may not be adequately taken up into or affecting the brain or blood. This is preliminary evidence and would need to be confirmed by future studies.

When looking at the genes from Table 1, we noticed that female mice on the high DHA diet had significant increases in the amount of myelin associated proteins,

specifically MBP, MAL, MAG, CLDN11, PLP1, MOG, and MOBP. These genes were all increased in the PFC of female mice on the high DHA diet as compared to the female mice on the low DHA diet. Conversely, these genes are decreased in human postmortem PFC of bipolar patients, depressed, and alcoholic patients. Other studies have also reported decreases in myelin genes in the postmortem brains of bipolar patients [94]. DHA may be exerting its effects by upregulating myelin production in oligodendrocytes, bringing neurotransmission back to normal levels.

Also of particular interest from the female list of genes is the upregulation of genes related to dopamine signaling. Particularly, DRD2, the gene producing the dopamine D2 receptor, and DRD1A, which encodes the D1 receptor A subunit, were both upregulated in female ST DBP KO mice on the high DHA diet compared to their counterparts on the low DHA diet. Studies have shown altered dopaminergic transmission in bipolar patients [13], so the upregulation of these receptors may represent the fine-tuning of dopaminergic signaling in the PFC of the female mice on the high diet, leading to modulated mood.

In addition to their involvement in neurological disease and neuron development, many of the top candidate genes for males are involved in gene expression regulation and cell signaling. Some of the top male candidate genes are associated with Reelin signaling. Interestingly, Reelin expression is regulated by dopamine responsive promoters [201]. Changes in dopamine signaling, which are seen in bipolar patients, could affect promoter epigenetics, leading to altered expression of genes involved in Reelin signaling, affecting neuronal outgrowth. In addition, transcription factors, such as SFPQ were increased in the PFC of male mice, which could further alter gene expression by binding to histone

deacetylases. Though there was no overlap between the genes changed in males verses females, pathways in which these genes are associated did overlap and it appears that DHA may be affecting more upstream proteins in the females and affecting their downstream effectors in males.

Bipolar disorder often occurs with co-morbid alcoholism in humans and our previous data showed that DBP KO mice drink significantly more alcohol than their WT littermates. Over a two week and four week period, male ST KO mice on the high DHA diet drank significantly less total ethanol than male ST KO mice on the low DHA diet. Similarly, over two weeks, P rats drank significantly less ethanol when on the high DHA diet compared to P rats on the low DHA diet, with a dose-dependent trend for P rats on a control diet. It would be interesting to do genetic testing on these mice, as was performed for the male and female mice in the other experiments to discover potential candidate genes for bipolar disorder with co-morbid alcoholism and possible blood biomarkers, as well. It would also be interesting to see if NST DBP KO mice, which model the depressive aspect of bipolar disorder, drink less alcohol with increased DHA in their diet. As with our previous observations, an increased number of animals needs to be tested to confirm our results. Nevertheless, these studies suggest a potential application of DHA supplementation in decreasing alcohol consumption.

For these studies, we wanted to ensure that responses to DHA could be observed, so we chose extremes diets, with trace amounts of DHA in the low diet compared to extensive amounts in the high diet. Indeed, in Western industrialized countries, the diet consists of high levels of saturated fat with a large omega-6/omega-3 ratio at 15:1 [202]. Though we were able to reach significance for the effects of DHA on behavioral

outcomes, a control diet with an average amount of DHA, as was used for the rat data, would also be informative and will be used in future studies.

Although our studies have yielded significant data, there are some limitations that are worth mentioning. One limitation is that our mouse model is a knockout of only one gene, however, bipolar disorder is likely induced by mutations or deletions of numerous genes. Nevertheless, we were able to model many aspects of the disease, with the mice showing depressed behavior at baseline, and manic-like, impulsive behavior when stressed. Another drawback is that we only performed two behavioral analyses on these mice. It would be interesting to see how they perform on memory and anxiety tests to see if there are deficits or changes in these outcomes, as cognition impairment and stress are linked to bipolar disorder [40, 203]. It would have also been helpful to test NST DBP KO and WT mice, as was performed in our previous study. In that study, NST DBP KO mice showed depressed behavior at baseline, and it would be interesting to know if DHA's anti-depressant effects can be seen in these mice in addition to the ST WT mice. Another restriction of our study is that we only looked at the PFC as the brain region to study changes in gene expression. It would also be informative to study the effects of DHA in other brain regions, such as the amygdala, that have been associated with bipolar disorder [204-205]. The fact that our genetic ranking is dependent on the research field, as it is based on present, published data, is another limitation of our study. In this sense, genes that have not yet been connected with bipolar disorder, but still have important roles in the disease, may be omitted from the list or ranked low due to lack of sources discovering this role. With this in mind, our CFG analysis will improve with time, as more significant evidence is being published, and roles of genes involved in the disorder are discovered. A

final concern of this study is that there were no overlaps between the genes on the male list and the female list. Perhaps DHA is affecting different genes of similar pathways in males and females, or the deletion of DBP affects the brains and blood of females in a different way than males. Indeed further studies need to be done on a larger cohort of mice to reach definitive conclusions.

Conclusion

DHA can potentially serve as a mood stabilizer for bipolar disorder patients based on our behavioral data in a mouse model of the disorder and molecular data obtained from the brain and blood of these animals. Likewise, DHA reduced alcohol intake in these mice and in an additional rodent model, the alcohol-preferring (P) rat. Although promising, our studies suggest further work is necessary to discover the potential of DHA as a mood stabilizer and alcohol suppressant in rodents, and its implications for humans with bipolar disorder.

CHAPTER III: IMPLICATIONS AND FUTURE DIRECTIONS

In this study we were able to further validate the DBP KO as a potential rodent model of bipolar disorder. We replicated the significant extremes in behavioral results between ST DBP KO and ST WT mice when fed a low DHA diet using forced swim and open-field movement tracking behavioral tests. However, when fed a diet high in DHA, there was no longer a significant difference between groups. Indeed, values fell between the extremes of the two groups, suggesting a balance of behavior. Under these conditions, the hyper-mobility of the ST DBP KO mice and the depressed mobility of the ST DBP WT mice were stabilized between the two extremes, indicating the possibility that DHA supplementation stabilized mood in these mice. In addition, the anti-depressive effects of DHA are stronger in female mice, while the anti-manic effects are stronger in male mice. However, a study using a much larger cohort of mice is necessary to confirm these conclusions. Unfortunately, we did not test NST DBP KO mice, which showed a depressive-like phenotype in our previous study. However, our ST WT mice, which also showed a depressive-like phenotype, demonstrate an anti-depressive response, especially for female mice, when on a high DHA diet. Perhaps DHA would have an anti-depressive affect on NST DBP KO mice as well, but future studies are needed to extrapolate to these mice.

From our microarray data, we were able to generate lists of numerous genes that were significantly changed in at least two out of three microarray experiments comparing ST DBP KO mice on a high verses low DHA diet. Among the genes changed by DHA treatment in the PFC of male mice were FOS, NDUFAB1, and COL16A1. These genes were changes in the opposite direction as reported for the postmortem brain of humans

with bipolar disorder. Similarly, the genes MBP, NEFH, GSN, PPP1R1B, CLDN11, MOG, QDPR, SYT2 MOBP, and PLP1 were changed in the opposite direction in the PFC of the female ST DBP KO mice on the high DHA diet as compared to the postmortem brain of humans with bipolar disorder. This indicates a potential reversal by DHA supplementation of gene expression levels abnormally altered in bipolar patients. In addition, Ingenuity analysis showed that the key functions in which a vast number of changed genes are involved include neurological disease and nervous system development for males and neurological disease, nervous system development, cell death, behavior, and genetic and psychological disorders for females, all of which are associated with bipolar disorder. A diet high in DHA may be causing the observed stabilization of behavior in our mice by affecting the expression of these genes.

Though upregulation of the AA cascade has been strongly linked to bipolar disorder, and has been shown to serve as a target of currently prescribed mood stabilizers (Figure 1), our genetic results did not show changes in genes in this cascade. However, DRD2, the gene producing the dopamine D2 receptor, was upregulated in female ST DBP KO mice on the high DHA diet compared to their counterparts on the low DHA diet. The AA cascade has been linked to this receptor; however, more studies need to be performed to confirm this *in vivo* [29]. D2 is a G-protein coupled receptor that is activated by dopamine, and through downstream effects, exerts largely inhibitory responses in neurons. D2 receptors can be postsynaptic or can also serve as presynaptic autoreceptors. As elevated extracellular dopamine levels have been correlated with manic episodes [206], perhaps elevated DHA levels induce upregulation of the D2 receptor, in effect reducing dopamine release. Interestingly, the DRD1A gene, which encodes the D1

receptor A subunit, is also upregulated in the PFC of the female mice on the high DHA diet. A positron emission tomography study on bipolar patients in the euthymic, depressed, and manic states showed that D1-receptor binding is significantly decreased in the frontal cortex of these patients, regardless of mood, compared to control [207]. In this regard one might speculate that DHA is restoring the expression of this receptor to a more normal level. The upregulation of these two receptors may underlie the fine-tuning of dopaminergic signaling in the PFC of the female mice on the high diet, leading to modulated mood. It would be interesting to determine if dopamine signaling is indeed increased in the brains of our ST DBP KO mice, and if DHA can decrease this release. It would also be informative to perform experiments similar to those performed by Rao et al. and determine if AA levels in the PFC of NST and ST DBP KO mice on the high DHA diet are modulated compared to mice on the low DHA diet [5]. Using radiolabeled fatty acid and quantitative autoradiographic analysis, we could determine AA and DHA turnover rates in the PFC of our mice, indicating whether or not the AA cascade plays a role in the behavioral and genetic expression outcomes.

Of further interest, numerous genes associated with myelin are increased in the female ST DBP KO mice on the diet high in DHA compared to those on the low DHA diet. Many of these changed genes were increased in the PFC region of the females on the high diet relative to the females on the low diet. Interestingly, reduced myelin staining and expression of oligodendrocyte-related genes were reported in the postmortem brain of patients with bipolar disorder [208]. Neuroimaging and neuropathological studies have shown reduced oligodendrocytes in PFC white matter and alterations in the tracts that connect the PFC to subcortical regions known to be involved in emotion [209]. Kato

suggests that this may be due to a vulnerability to stress and death of some neurons, an hypothesis supported by the reduced density of neurons in brain areas such as the PFC, anterior cingulated gyrus, and hippocampus of bipolar patients [13]. To further test this, immunohistochemistry could be performed on brain slices from DBP KO mice, staining for myelin associated genes, to assess a deficit in myelin in the PFC or other brain regions of these mice compared to WT mice.

A potential point of concern for our male gene expression data is that there is no overlap with those female genes that changed by criterion. However, many of the biological and canonical pathways for these genes did overlap. Perhaps the affect of DHA reaches the same outcomes for male and female mice, while targeting different genes to reach these same results. As we had expected, many of the genes changed in expression for males are involved in neurological disease, neuron development, and cell growth. Of further interest, many of the candidate genes are involved in gene expression regulation and cell signaling, specifically Reelin signaling. In conjunction with this finding, a study on rat brains showed significant alterations in the mRNA levels of Reelin associated genes when the rats were treated with mood stabilizers [210]. Of particular intrigue, Reelin expression is regulated by dopamine responsive promoters [201]. Bipolar patients indeed have alerations in dopamine signaling, which thus could affect promoter methylation or acetylation. This could lead to altered expression of genes including those involved in Reelin signaling, affecting neuronal outgrowth, axonal branching, and synaptogenesis [211]. Genes serving as transcription factors, such as SFPQ were also increased in the PFC of male mice, which could further alter gene expression. Based on

these findings, perhaps DHA is affecting more upstream proteins in the female mice and downstream substrates in the male mice.

Another interesting aspect of our study is that our mice were stressed via acute and chronic stressors. Indeed, mood and anxiety disorders are often co-morbid in humans [212] and several of the genes in our lists, especially for male mice, have been linked in humans to anxiety and stress, such as CDKN1C, PRKAR2B, CRYAB, FOXO3A, DRD1A, FOS NDUFAB1, ARF3, HSPA4, and UBE2D2. Studies on human volunteers have found reduced plasma adrenaline and noradrenaline levels after omega-3 fatty acid supplementation containing DHA and EPA for two months compared to placebo-treated controls [213]. Such findings suggest that omega-3 fatty acid supplementation may decrease the activation of the hypothalamic-pituitary-adrenal axis, which is related to anxiolytic effects [212]. It would be interesting in future studies to test ST DBP KO mice on a high verses low DHA diet on anxiety-related behavioral tests such as elevated plus maze, radial arm maze, and social interaction tests, to determine if DHA is able to lower anxiety-related outcomes for these tests as well.

In addition to the behavioral and genetic data, we were able to show that ST DBP KO mice on a high DHA diet drank significantly less total alcohol over a two week and four week period. Interestingly, when the acute stressor was added on day 21, the significance between groups was lost. Perhaps DHA works at suppressing alcohol intake up to a certain level of stress, but during extreme, acute stress, can no longer do so. Tests were also performed on a rat model of alcoholism and produced a dose-dependent trend for DHA treatment, with significant difference reached between high and low DHA diets. This not only supports our alcohol data for our mouse model, but also suggests that DHA

could be of benefit for suppressing alcohol consumption even without co-morbid bipolar disorder. It would be informative to provide a cohort of ST and NST DBP KO mice a drug that could serve as a positive control, such as disulfiram, which is an FDA approved alcohol suppressant [214]. We would compare alcohol consumption in these mice to mice without disulfiram but with DHA in the diet and to a negative control that was not given disulfiram or DHA. This would allow our laboratory to determine if DHA could be as effective as a presently prescribed medication for suppressing alcohol consumption, without causing the adverse side-effects sometimes induced by this medication.

For our studies, we wanted to model the low omega-3 fatty acids diets of the Western hemisphere verses the high omega-3 fatty acids diets of countries such as Japan, with individuals in Japan eating eight times the amount of omega-3 fatty acids ingested by the average American in the US per year [215], so we used diets with very low or very high DHA levels. It may also be beneficial to study the effects of a control diet on our mice, as was given to the alcohol-preferring (P) rats. This would provide dose-dependent information for a DHA effect, as well as a standard by which to compare the extreme groups. Moreover, it would be beneficial to offer diets consisting of increasing levels of DHA, as the high diet provided ~0.035 g of omega-3 fatty acid to the mice per day, which is the equivalent of 90 g daily of omega-3 fatty acid to an average-size human adult. Since this is quite an excess to the recommended dose of 500mg/day for heart patients [216], testing cohorts of mice with increasing amounts of DHA in their diet would be informative, as the optimal therapeutic amount could be determined.

Another potential addition for our studies may be the inclusion of the omega-3 fatty acid EPA in the high DHA diet. Like DHA, EPA levels are reduced in the

postmortem brains of bipolar patients [217]. In addition, EPA directly antagonizes membrane AA and reduces PGE_2 synthesis, thereby balancing immune and inflammatory function [6] (Figure 2). In fact, preliminary studies have shown beneficial effects of increased EPA in the diet for patients with psychiatric disorders [24, 26].

Once a reasonable, therapeutic dose of DHA supplement per day is determined for DBP KO mice (and potentially with EPA as well), and the results have been successfully replicated using large cohorts of animals, clinical trials could be considered on patients with bipolar disorder. Indeed, studies from other groups have reported beneficial effects of omega-3 fatty acid supplements for bipolar patients [60, 218].

One specific population that could benefit from DHA supplements are adolescents at high risk of developing bipolar disorder due to genetic and environmental factors. Indeed, a study by Amminger et al. gave adolescents at ultra-high risk for psychosis 480 mg of DHA a day, in addition to other PUFAs, or placebo for 12 weeks [24]. They showed that adolescents taking the DHA supplements had a significantly lower number of transitions to psychotic disorder than their placebo-treated counterparts (5% transition compared to 28%). A similar study could be performed on adolescents at high risk for bipolar disorder to see if DHA could prevent the transition to the disease. DHA would be an excellent candidate for these individuals, as it does not have clinically relevant adverse side-effects, and the use of medications for bipolar disorder is controversial for at-risk teens [15].

Another population of individuals who could greatly benefit from a healthy mood-stabilizing supplement is pregnant women with bipolar disorder. This disorder usually develops in late adolescence and early adulthood, which places its onset during

the prime reproductive years for women. A study performed on over 11,000 pregnant women found that, unlike mood stabilizing drugs that are currently prescribed for bipolar patients, consumption of food high in omega-3 fatty acids did not cause harmful effects to their offspring [199]. They discovered that maternal seafood consumption of more than 340 g per week provided beneficial effects on child development, whereas a diet consisting of less than 340 g of seafood per week did not protect children from adverse outcomes, such as suboptimum scores for prosocial behavior, communication, fine motor, and social development. They concluded that the risks from the loss of omega-3 fatty acids were greater than the risks of potential exposure to trace contaminants in 340 g or more of seafood eaten on a weekly basis. In addition, maternal DHA status declines in pregnant women after the second trimester [219]. Studies in our DBP KO mice could be performed where pregnant NST and ST DBP KO mice are given a diet high in DHA and developmental, behavioral, and genetic effects would be compared to NST and ST DBP KO pregnant mice given a low DHA or control diet. Another group of pregnant KO mice would be given a control diet with lithium to serve as a positive control of mood stabilization and potential birth defects [16]. Once confirmed in rodents, clinical trials could be performed where DHA supplements were given to pregnant patients with bipolar disorder to see if mood was stabilized, without harming the fetus.

Based on our studies, a final cohort that may benefit from DHA supplements is the 40-60% of bipolar disorder patients who have co-morbid alcoholism [220]. Animal studies have shown that prolonged alcohol consumption leads to a reduction in brain DHA levels [221-222], and our studies on two different rodent models showed a significant decrease in alcohol consumption with DHA diet supplementation. Current

drugs prescribed as alcohol suppressants have adverse side-effects [214], and DHA could provide a healthier supplement for these individuals, without causing negative side-effects. Our evidence is preliminary, however, and much further study needs to be accomplished before it can be translated to humans.

Overall, our studies with DBP KO mice reveal the potential of the DBP gene as a biomarker for bipolar disorder and support the DBP KO mice, under non-stressed and stressed conditions, as a strong candidate model of the disorder. This model is limited, however, since bipolar disorder is likely caused by a compilation of mutated genes. Nevertheless, the DBP KO mouse serves as a good model in which to test bipolar disorder mechanisms and potential mechanisms of drug and supplement treatments. Indeed, based on our studies, in addition to the vast number of previous studies performed by other laboratories, supplements of the omega-3 fatty acid, DHA, may have mood stabilizing capabilities and this potential warrants further research.

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CURRICULUM VITAE

Natalie J. Case

EDUCATION

Master of Science in Medical Neuroscience, May 2010
Indiana University, Indianapolis, IN

Bachelor of Science in Biology, minor in psychology, May 2007
Taylor University, Upland, IN
Summa Cum Laude

RESEARCH EXPERIENCE

Research Assistant – Laboratory of Neurophysiology
Indiana University School of Medicine, Medical Neuroscience Program, Indianapolis, IN
August 2009 – May 2010
Researched under the direction of Dr. Gerry Oxford to study the methods by which post-receptor signaling elements in the cell can induce differential downstream effects in dopamine signaling

Research Assistant – Laboratory of Neurophenomics
Indiana University School of Medicine, BioMedical Gateway Program, Indianapolis, IN
September – December 2007, May 2008 – July 2009
Researched under the direction of Dr. Alexander Niculescu to study the behavioral and genomic effects of a diet high in omega-3 fatty acids in animal models of bipolar disorder and alcoholism

Student Researcher (Rotation) – Laboratory of Neuroimaging
Indiana University School of Medicine, BioMedical Gateway Program, Indianapolis, IN
March 2008 – May 2008
Researched under the direction of Dr. Andrew Saykin to learn neuroimaging techniques and study MRI scans to determine brain changes caused by mild cognitive impairment

Student Researcher (Rotation) – Laboratory of Medical and Molecular Genetics
Indiana University School of Medicine, BioMedical Gateway Program, Indianapolis, IN
January 2008 – March 2008
Researched under the direction of Dr. Xin Zhang to discover the role of the Pax6 gene in the development of the mouse fetus

Student Researcher (Undergraduate Research) – Biotechnology Program
Ball State University, Muncie, IN
January 2006
Researched under the direction of Dr. Susan McDowell to test factors that influence *Staphylococcus aureus* infection of human umbilical vein endothelial cells

HONORS AND AWARDS

Awarded Educational Enhancement Grant
Indiana University School of Medicine, Indianapolis, IN
Spring 2009

Awarded Second Place Poster Presentation, First Annual Research Day
Indiana University Purdue University Indianapolis, IN
Spring 2009

Awarded Stark Scholar Fellowship
Indiana University Medical Neuroscience Program, Indianapolis, IN
August 2008 – August 2009

Awarded University Fellowship and Travel Grant
Indiana University BioMedical Gateway Program, Indianapolis, IN
August 2007 – August 2008

Graduated Summa Cum Laude,
Taylor University, Upland, IN
Spring 2007

Inducted into Alpha Chi
Taylor University, Upland, IN
Fall 2005

Named to Dean's List,
Taylor University, Upland, IN
Fall 2003, spring 2004, fall 2004, spring 2005, fall 2005, spring 2006, fall 2006, spring 2007

National Collegiate Award Winner, United States Achievement Academy
August 2005 – August 2006

ASSOCIATIONS, CERTIFICATIONS AND SKILLS

- Member of the American Association for the Advancement of Science
- Certified by the Institutional Animal Care and Use Committee at the Indiana University School of Medicine to perform research on animals
- Certified through the Indiana University Protection of Human Research Participants Certification Test to perform research on human subjects
- Experienced in lab techniques, specifically Western blotting, PCR setup, mouse behavioral analysis tests, mouse and rat colony maintenance, mouse genotyping, ear tagging, and tail clipping, cell culture maintenance, whole-cell patch clamping, DNA and RNA extraction, gel electrophoresis, microarray gene expression analysis, immunohistochemistry, sectioning frozen tissue, and preparing slides

PRESENTATIONS

Poster Presenter – Society of Biological Psychiatry 64th Annual Scientific Convention
Vancouver, Canada
May 2009

Poster Presenter – 1st Annual Research Day
Indiana University Purdue University, Indianapolis, IN
April 2009

Poster Presenter – Recruitment Weekend, Indiana University BioMedical Gateway Program
Indiana University School of Medicine, Indianapolis, IN
January 2009

PUBLICATIONS IN PROGRESS

"Omega-3 fatty acids as treatments for psychiatric disorders: phenomic, genomic and blood biomarker studies in animal models." H. Le-Niculescu, N.J. Case, S. Patel, L. Hulvershorn, R. Bell, H.J. Edenberg , J.I. Nurnberger , Z. Rodd and A.B. Niculescu

TEACHING EXPERIENCE

Teaching Practicum in Health and Rehabilitation Science
Indiana University School of Medicine, Indianapolis, IN
Spring 2009

Assistant Director of Heritage Summer Players
Heritage Christian School, Indianapolis, IN
Summer 2007

Teaching Assistant of Animal Physiology
Taylor University, Upland, IN
February 2007 – May 2007

Teaching Assistant of Genetics
Taylor University, Upland, IN
August 2005 – December 2005

OTHER WORK EXPERIENCE

Administrative Assistant for the Administrative Office of the Student Center
Southern Illinois University, Carbondale, IL
June 2005 – August 2005

Banquet Server for Creative Dining Services
Taylor University, Upland, IN
Spring 2004 – Spring 2007

COMMUNITY SERVICE

Small Group Co-Leader, Trinity Church, Indianapolis, IN
Fall 2009 – May 2010

Student Mentor for incoming students in the Indiana University BioMedical Gateway Program, Indianapolis, IN
Fall 2008 – May 2010

Student Ambassador for the Indiana University BioMedical Gateway Program, Indianapolis, IN
January 2009

Youth Conference Small Group Leader at Taylor University, Upland, IN
Spring 2004, spring 2006

New Student Orientation Small Group Leader at Taylor University, Upland, IN
Fall 2004